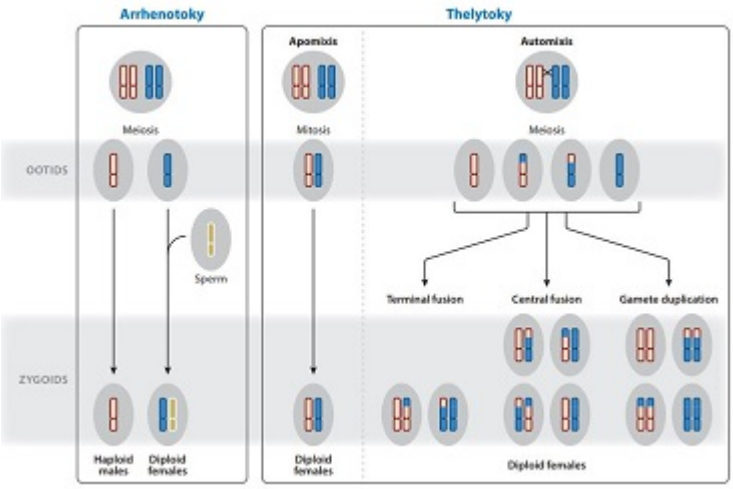


Parthenogenesis in insects: synthesis

SEPTEMBER 11, 2018

by Benoit GILLES

In order for them to multiply, nature has endowed living organisms with a wide diversity of reproductive systems. In insects, one of these strategies is **parthenogenesis**. This is based on the development of individuals from unfertilized gametes, thus without the need for fertilization. This strategy is interesting for more than one reason: it is evidenced by the declination of a multitude of forms (thelytokia, arrhenotokia and deuterotokia) and its appearance on multiple occasions during evolution, within unrelated and phylogenetically distant taxa and species.

Description of the different types of parthenogenesis

I) Thelytokia

Corresponding to parthenogenesis *sensu stricto*, thelytokia is characterized by the fact that all the unfertilized eggs emitted by a female insect in turn only produce a female diploid progeny.

Thelytoic parthenogenesis can result in two distinct genetic systems: one based on mitosis (apomixis) and the other on meiosis (automixis).

• L'**apomixie** is the simplest system: female offspring are genetically similar to the mother, the absence of meiosis prevents any chromosomal rearrangement (genetic mixing). Mother and daughter can thus be considered as clones

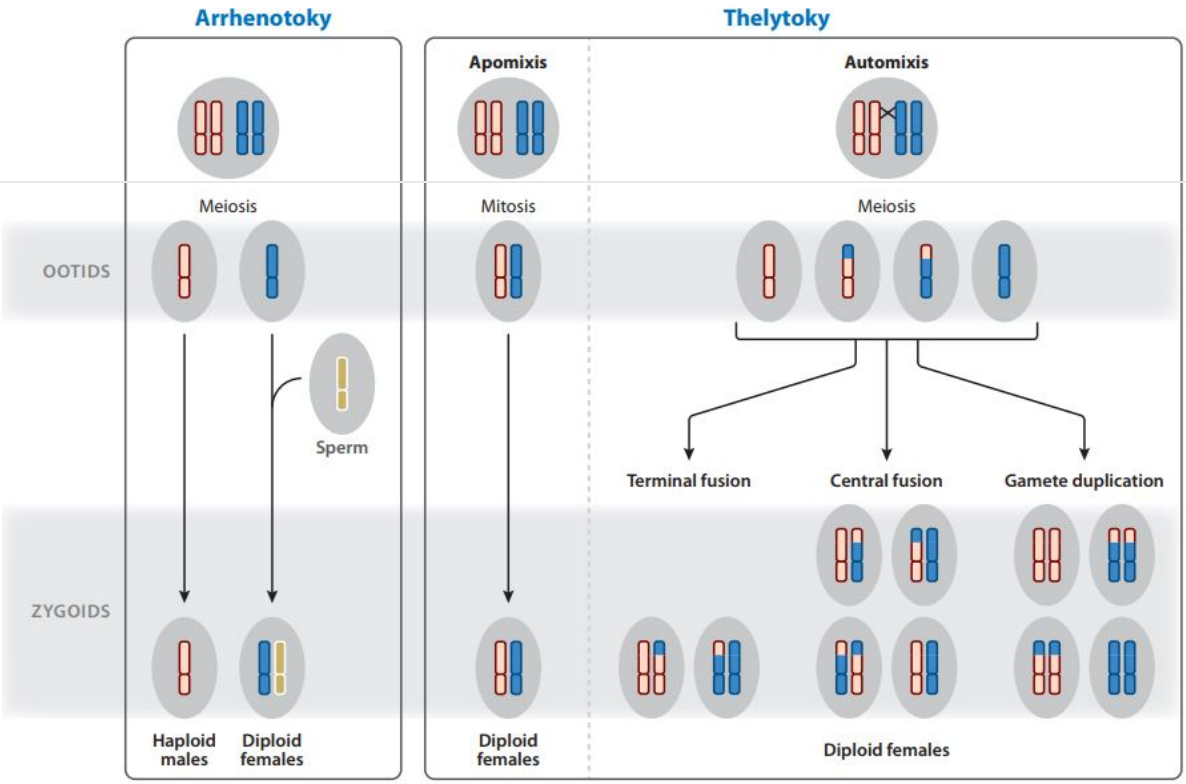
• L'**automixie** involves the meiosis process, diploidy is restored without the contribution of a gamete (without fertilization). This process can be carried out in several ways: 1) duplication of the genome before meiosis, creating a cell with 4n chromosomes (4 sets of chromosomes); 2) fusion of the two nuclei during meiosis; 3) a haploid nucleus resulting from meiosis is duplicated by mitosis to fuse again with itself

II) Arrhenotokism

In arrhenotokic parthenogenesis, progeny from unfertilized eggs consist only of male individuals (opposite system to thelytokia). There are two distinct genetic systems: 1) **haplodiploid**, males are haploid (1 set of chromosomes) and females are diploid (2 sets of chromosomes); 2) **diploid**, males and females are derived from diploid eggs as for thelycan parthenesis.

III) Deuterotokia

Deutherotokic parthenogenesis produces unfertilized eggs from both males and females.



Parthenogenetic reproduction modes (Source : Rabeling & Kronauer, 2013)

Appearance of parthenogenesis

A parthenogenetic system can appear in various ways. The most singular case is based on the interaction between certain groups of insects and endosymbiotic bacteria such as *Wolbachia*, *Rickettsia* and *Cardinium* – the most widely represented being *Wolbachia pipientis* – causing cytoplasmic incompatibilities causing either the cessation of the development of diploid embryos, or the thelytokia, or the feminization of males, their death. These bacteria often play a role in sex conversion and modify the sex ratio in favour of females.

Many insect species are monosexed (their population is composed of only one sex). These species occur in isolated environments such as islands or high-altitude regions. They perform a **geographic parthenogenesis**, demonstrating the adaptive power of this type of reproduction.

>Parthenogenesis in hemimetaboles (or heterometaboles)

Hemimetazole insects federate species whose metamorphosis is said to be incomplete: the adult stage is reached gradually during the development cycle by successive moulting, the larvae possessing most of the attributes of adults, except sexual and parasexual organs (***link article*** **(https://passion-entomologie.fr/metamorphosis-in-insects/)**).

Odonata (dragonflies) : only the species *Ischnura hastata* (Caenagrionidae), found in the Azores, has been described as parthenogenetic (thelytoic type).

Orthoptera (locusts, crickets and grasshoppers) : the species *Locusta migratoria* and *Schistocerca gregaria* have the ability to spontaneously generate female offspring from unfertilized eggs: **thycoparthenogenesis**. Haploid gametes become diploid gradually during development. For *Loxoblemmus frontalis*, the only species in the Gryllidae family to practice parthenogenesis, thelytokia is induced by the presence of bacteria *Wolbachia*. It is without intervention of *Wolbachia* in grasshoppers *Saga pedo* (Tettigonidae).

Phasmatodea (Phasms) : parthenogenesis is quite common. For example, the genus *Timema*, endemic to California, is composed of 5 species all related and descended from the same lineage. However, rare fertile males could be collected, their presence being estimated at less than 0.2% of the population. The sexual determination of phasms is based on a system where individuals carrying a pair of autosome chromosomes (XX) are female and those with only one copy of the X are male (XO), (in humans the system is XX-XY), males develop *via* spontaneous loss of the X chromosome during oogenesis. Other species also reproduce only by parthenogenesis such as *Bacillus rossius* and *Clonopsis gallica* (species found in southern France).

Phasme du genre *Timema* – Santa Barbara – Californie (Source : **Alice Abela (https://bugguide.net/node/view/1051212)**)

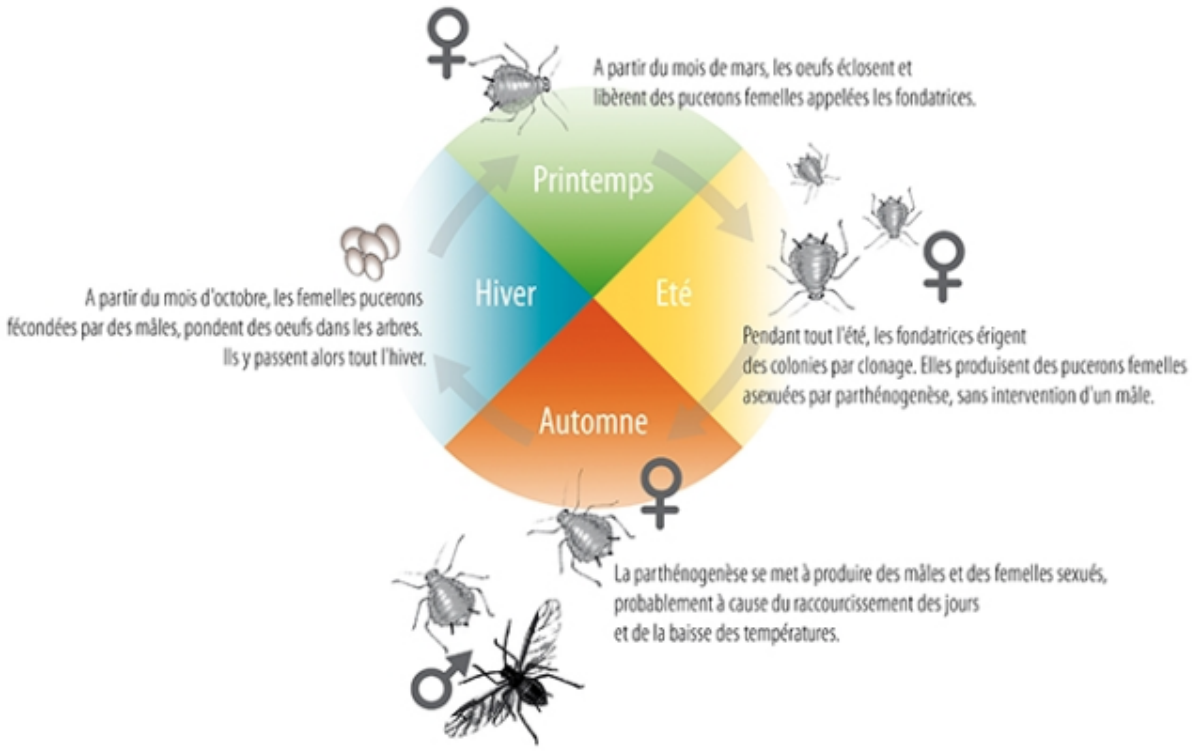
Blattoptera (formerly **Isoptera)** (termites) : several groups optionally use thelycan parthenogenesis through a ploidy restoration process similar to that found in *Reticulitermes speratus* and *R. virginicus* (automiscie). This type of termites is known to harbour *Wolbachia*, whether the colonies are parthenogenetic or bisexual, suggesting that there is no correlation between the two reproductive modes.

Mantodea (mantes) (***lien (https://passion-entomologie.fr/the-mantodea-synthesis-on-these-insects-by-nicolas-moulin/)***) : only two species, *Miomantis savignyi* and *Bruneria borealis,* carry out parthenogenesis (thelytoque), mandatory parthenogenesis in *B. borealis*.

Hemiptera (cicadas, aphids and bedbugs) : this order offers an abundant diversity of parthenogenetic systems within a large number of families such as Aclerdidae, Diaspididae, Aphididae or Anthocoridae. For example, several species of Fulgores (Delphacidae) belonging to the genera *Delphacodes* and *Ribautodelphax* use thelytokia and pseudogamy (females mate with males but the offspring are entirely female). The intervention of *Wolbachia* in the parthenogenesis process has been demonstrated in

Delphacodes kuscheli but not in other species. A multitude of forms of parthenogenesis occur between species of the Coccidae and Diapsididae families: arrhenotokism with males that may be diploid or haploid, and deuterotokism.

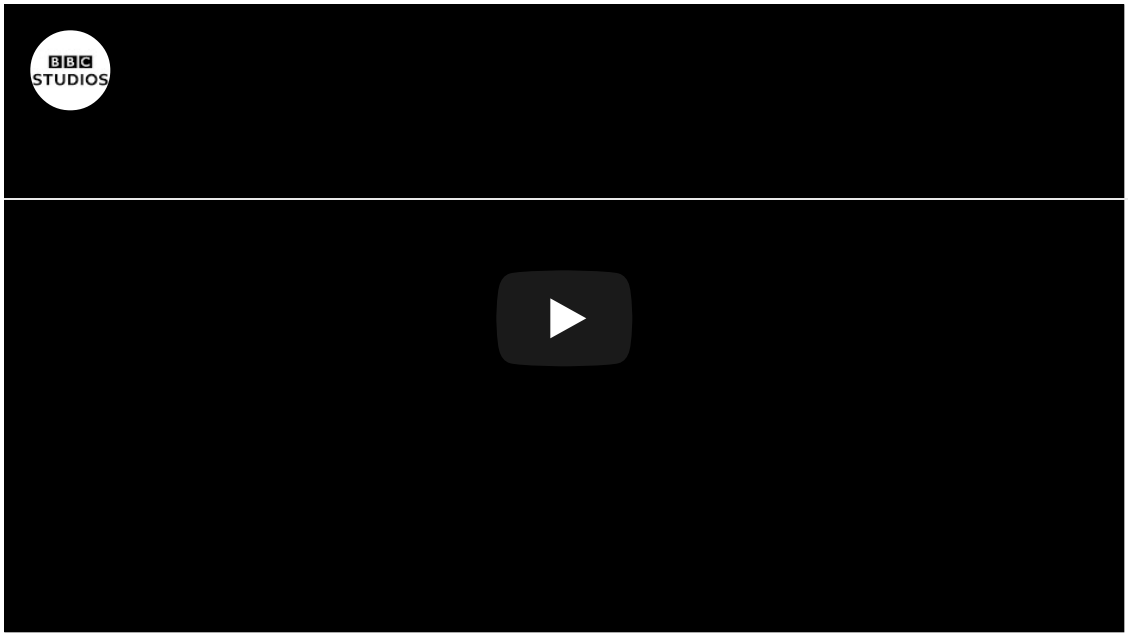
Some aphid species (Aphidimorpha) have a cyclic reproductive mode, changing from bisexual to parthenogenetic depending on the season. Thus, in spring, a female aphid (founder) multiplies by parthenogenesis in order to rapidly colonize the environment, then, in autumn, reproduction becomes sexual, resulting in the production of fertilized eggs that overwinter in the vegetation.



Aphid life cycle (Source:)

With regard to “real” bedbugs, there are no cases of parthenogenesis revealed. However, two species have optional use of telytokism: *Calliodis maculipennis* (neotropical species of the Anthociridae family) and *Campyloneura virgula* (Miridae). Surprisingly, a parthenogenetic population of *C. maculipennis* located on the island of Trinidad has been discovered, while the populations of the mainland, Mexico and Guyana, are gendered.

Life cycle of the aphid (*english*)



Parthenogenesis in holometabolas

The holometabol group characterizes species using a so-called complete metamorphosis phase where the passage to adulthood requires a chrysalis (butterfly) or pupa (flies) phase during which the larvae are totally transformed (***lien article* (https://passion-entomologie.fr/metamorphosis-in-insects/)**).

Hollo-metabolas make up the vast majority of insect species diversity: 800,000 species divided into 11 orders such as Diptera, Coleoptera, Lepidoptera or Hymenoptera.

The Hymenoptera (wasps, bees, ants), with nearly 150,000 species described, constitutes one of the most diversified insect orders. Parthenogenesis is common to all species of the order, thus encompassing the greatest diversity of types of parthenogenesis. The most common and ancestral is arrhenotokism combined with haplodiploidy. Females thus have the ability to fertilize or not their eggs and can adjust the sex ratio of their offspring: a diploid egg will give a sterile female or worker and a haploid egg a male (*see illustration below)*. However, mechanisms may vary between taxa.

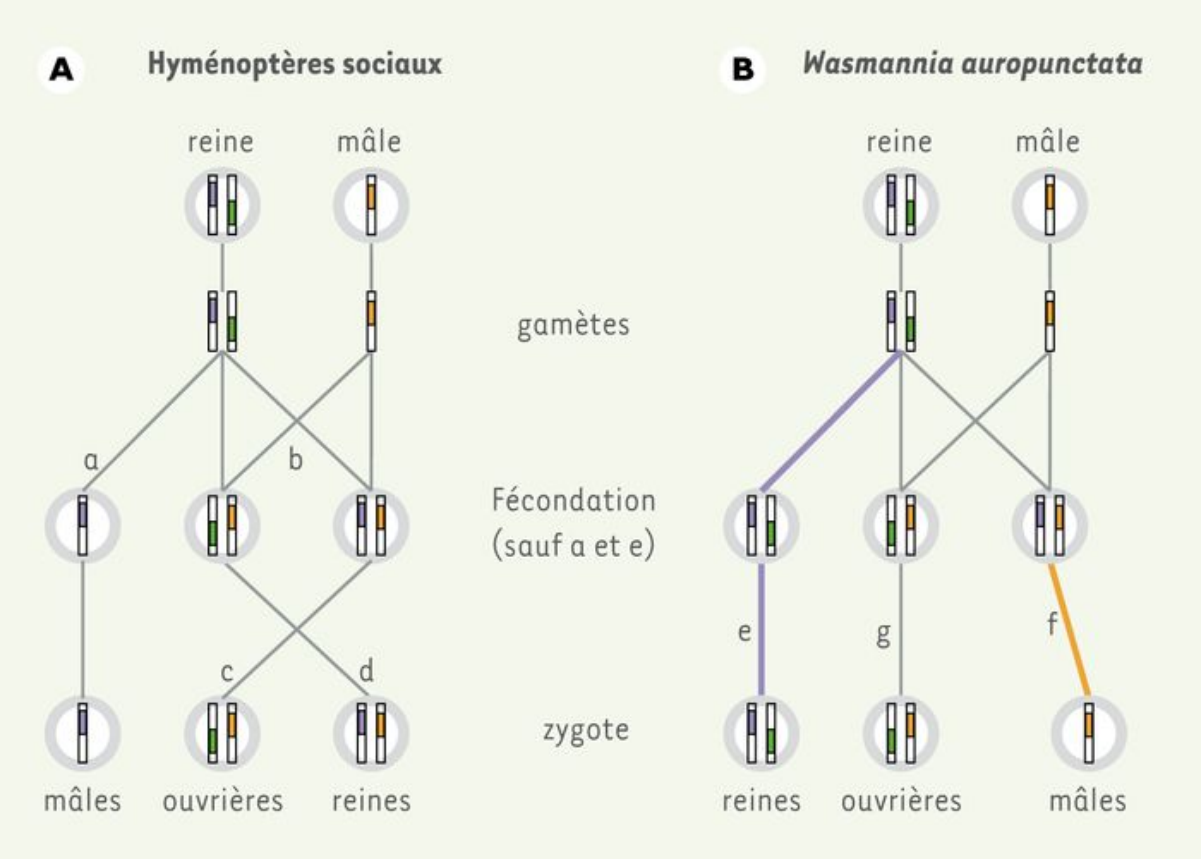
The transition from arrhenotopic to thelytoic parthenogenesis is relatively frequent due to the absence of a sexual chromosome making it possible to restore diploidy through an apomictic or automictic process. For example, the species *Diplolepis eglanteria* (Cynipidae) is a small wasp that uses apomixis, and *Apis mellifera capensis* a, on the other hand, uses self-mixing.

Cataglyphis hispanica, *Paratrechina longicornis*, *Vollenhovia emeyri* and *Wasmannia auropunctata* are derived from sexual reproduction while the new queens come from parthenogenetic thelycan eggs. The study of the reproductive mode of ***Wasmannia auropunctata*** revealed a unique case of a dual parthenogenetic system (arrhenotokic and thelytok). It was discovered in 2005 that males, derived from fertilized eggs,

expressed only the paternal genome, with the maternal genome disappearing (except the mitochondrial genome) through a mechanism that remains partly unknown. This process indicates that male offspring are cloned (*see illustration opposite*).

Queen *Wasmannia auropunctata* and sterile workers – As with many ants species, the queen is more imposing than the workers (Source : **Alex Wild (https://www.alexanderwild.com/Ants/Taxonomic-List-of-Ant-Genera/Wasmannia/i-2sssQXb/A)**)

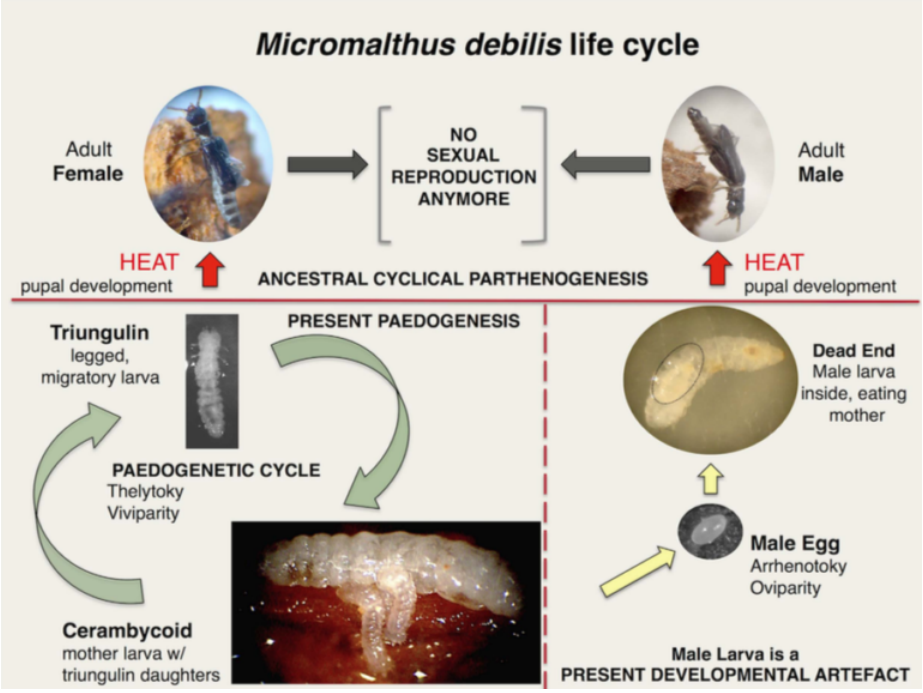
So far as these workers are sterile, this sexual reproduction does not lead to the mixing of male and female genomes in the next generation. This lack of gene flow between males and females leads to genetic differentiation and separate evolution of the two genomes. This raises the question as to the classification of a possible distinction between these two sexes as two distinct species, one of which would only consist of males! In addition, males can be considered as parasites exploiting females, where the production of sterile workers ensures the protection and supply of the colony.



"Classic" reproductive system of social hymenoptera (A) and ant *Wasmannia auropunctata* (B) (Source: ***Erudit.org*** **(https://www.erudit.org/fr/revues/ms/2005-v21-n11-ms1020/011950ar/)**)

At 30% of parasitoid wasp species (***article link*** **(https://passion-entomologie.fr/two-wasps-a-caterpillar-and-a-cabbage-leaf/)**) Cynipidae and Chalcidoidea, thelytokia is caused by endosymbiotic microorganisms of the genus *Wolbachia*, *Cardinium* and *Rickettsia*.

The order of Coleoptera accounts for 30% of insect species, or 380,000 species of which only 600 species (20 families) are parthenogenetic. Thelytokia is more widespread than arrhenotokia: it is found in Alexiidae, Anobiidae, Cerambycidae, Dermestidae, Elateridae, Hydrophiliidae, Passalidae, Sphindidae or Staphylinidae, among others. The case of *Micromalthus debilis* is unique in its kind, the thelytokia is pushed to the extreme because the sterile males have almost disappeared from the populations (*lire* ***this article*** ***(https://passion-entomologie.fr/the-incredible-life-cycle-of-micromalthus-debilis/)***).



Life cycle of *Micromalthus debilis* (***Link article*** **(https://passion-entomologie.fr/the-incredible-life-cycle-of-micromalthus-debilis/)**)

So, the species *Reesa vesopulae* (Dermestidae), present throughout the nearctic region, is strictly parthenogenetic, as are the majority of North American populations of *Cis fuscipes* (Ciidae) and *Aelus mellillus* (Elateridae). In Chrysomelidae, the species *Bromius obscurus* is represented by diploid bisexual populations in North America and other apomictic triploids in Europe. The two European weevil species (Curculionidae), *Polydrus mollis* and *Otiorhynchus scaber* are mainly thelytoics, although some populations are bisexual diploids in small localities.

In Lepidoptera, despite more than 170,000 species described, only two dozen species practice parthenogenesis. These species are mainly Lymantriidae and Psychidae (11 species). There is a gendered form of *Dahlica triquetrella* in Central Europe, while several diploid and tetraploid thelytok populations are widespread throughout Europe and North America.

Flies (**Dipteran Orders**), parthenogenesis has appeared in at least 11 families with more than 150 000 species : Chironomidae, Hybotidae, Agromyzidae, Cecidomyiidae, Psychodidae, Sciaridae, Ctenostylidae, Lonchopteridae, Simuliidae, Ceratopogonidae and Chamaemyliidae. Half of the Chironomidae taxa studied appear parthenogenetic. This strategy seems to have been selected to survive extreme environmental conditions, particularly cold (altitude and latitude), such as *Eretmoptera murphyi* (Antarctica) and *Micropsectra sedna* (Canada). Others, such as *Cladotanytarsus aeiparthenus* and *Paratanytarsus grimmii*, live in polluted or acidic waters.

Troglocladius hajdi and *Lymnophyes minimus* living in the Gough and Nightingale Islands south of the Atlantic Ocean, or *Monopelopia caraguata*, *Phtytelmatocladius delarosai* and *Polypedilum parthenogeneticum* which live in small puddles (water accumulated in the leaf axil, trunk cavity, etc.)) located on terrestrial plants: **phytotelme**, from ancient Greek *phyto* – plant *telma* – pond.

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Chapter 5
Reproduction of Earthworms: Sexual Selection and Parthenogenesis

Darío J. Díaz Cosín, Marta Novo, and Rosa Fernández

5.1 Introduction

Earthworms are generally considered to be cross-fertilization hermaphrodites (i.e., using reciprocal insemination, transferring, and receiving sperm in the same copulation). Although not all earthworms use this reproductive strategy, the best known species, *Lumbricus terrestris*, is a cross-fertilization hermaphrodite and this strategy seems to be the most widespread in earthworms. Nevertheless, cases of self-fertilization have been reported in earthworms; Domínguez et al. (2003) discussed that *Eisenia andrei* individuals bend themselves, allowing their spermathecal pores to contact the ventral zone of their clitellum. The sperm is then transported from the male pores to the spermathecae. This finding explains why 33% of isolated individuals in this study produced viable cocoons.

However, hermaphroditism is not the only reproductive mechanism and more parthenogenetic earthworms are being discovered all the time, most of which are polyploid. Parthenogenetic reproduction is very frequent in the family Lumbricidae, with more than 30 parthenogenetic species occurring in North America (Reynolds 1974). Parthenogenesis has also been reported in families such as Megascolecids, but has not been observed in other families, including Glossoscolecids.

"Asexual" reproduction by means of bipartition, stolonisation, budding, or similar processes has not been observed in earthworms and their ability to regenerate is limited. There are several reproductive models: discontinuous, semicontinuous, or continuous. In *Hormogaster elisae*, male and female gametogenesis are synchronized, beginning in autumn and ending in the summer. Male funnels are full of spermatozoa and the spermathecae contain spermatozoa throughout the year, but

D.J. Díaz Cosín (✉), M. Novo, and R. Fernández
Departamento de Zoología, Facultad de Biología, Universidad Complutense de Madrid, Ciudad Universitaria, 28040 Madrid, Spain
e-mail: dadico@bio.ucm.es

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two peaks of reproduction have been observed, with the largest peak occurring in the spring and the second peak occurring in autumn (Garvín et al. 2003).

An excellent description of the earthworm reproductive system can be found in general zoology volumes and monographs such as Jamieson (2006), so it will be only succinctly described in the present chapter. Earthworms are usually hermaphrodites in which the testes and ovaries are accompanied by a series of organs with a male or female function. The female components typically include the ovaries (generally one pair in the 13th segment), ovisacs (in the 14th segment), oviducts, female pores (in the 14th segment), and spermathecae (of variable position and number). Male components typically include the testes and male funnels (in most cases, there are two pairs in the 10th and 11th segments and singularly a single pair in the 11th segment), seminal vesicles (of variable number, with 2–4 occurring in segments 9–12), deferent ducts, and male pores surrounded by atrial glands that are more or less developed. Other organs, such as testicular sacs (*Lumbricus* and *Octolasium*), accessory glands (prostates), or the thecal glands associated with the spermathecae, may also be present.

Some of the external reproductive organs, such as the clitellum, tubercula pubertatis, and sexual papillae, are developed at sexual maturity. The sexual papillae include modified genital chaetae and chaetal glands, which could be used to inject substances into the partner (see Sect. 8.2.2).

The union during copulation, which could last between 69 and 200 min in *L. terrestris*, is secured by tubercula and quetae. Copulation can occur at the surface in epigeic and anecic earthworms, which increases the depredation risk, and also occurs in deeper layers of the soil in the case of endogeic species. The more primitive type of copulation seems to be a simple juxtaposition of the male pores of one individual and the spermathecal pores of the other, with the direct transfer of spermatozoa. The presence of a penis has been observed in some cases, which in reality seems to be just an elevated papilla, as in the case of some *Pheretima* species.

In most of the species in the Lumbricidae family and in other families, the clitellum moves backwards and seminal groves are developed from the male pores to the tubercula pubertatis. Spermatozoa flow through the seminal groves to get into the partner's spermathecae pores. Details of sperm transfer are not well known with the exception of a few species such as *Pheretima sp*., in which, according to Tembe and Dubash (1961), the sperm appears to be transferred sequentially, passing first to the anterior spermathecae and later to the posterior ones.

Bouché (1975) indicated that spermatophores have been observed in more than 20 species of lumbricids. Spermatophores are small capsules that adhere to the body wall and can be iridescent and full of spermatozoa. Their function is not clear. It has been suggested that the spermatophores may play a role in sperm transfer (Edwards and Bohlen 1996), thus avoiding sperm digestion in the spermathecae and fertilizing the ova during cocoon formation Michiels (1998). Nevertheless, Monroy et al. (2003) showed that spermatophores have no effect on the reproductive success of *Eisenia fetida* and were not able to demonstrate the specific function of these capsules.

Complex precopulatory behaviors have been described in partner selection in some species, including *L. terrestris*, in which individuals perform visits to their neighbors' burrows (Nuutinen and Butt 1997; Michiels et al. 2001, see Sect. 8.2.1). Development is direct in earthworms. Fertilization occurs within cocoons and one or more juveniles are produced for each cocoon.

The presence of parthenogenesis in earthworms was first observed many years ago, thanks to the contributions of authors such as Omodeo (1951), Casellato (1987), Jaenicke and Selander (1979) and Victorov (1997), among others.

Reynolds (1974) pointed out that in North America 35 species are anphimictic, 11 probably sexual, 4 facultative parthenogenetic, 1 possibly parthenogenetic, and 30 parthenogenetic. Casellato (1987) cited 25 parthenogenetic species or subspecies (12 of which had even ploidy numbers and 13 of which showed odd ploidy) and Victorov (1997) pointed out that in Russia, the number of polyploids almost equals the number of diploids, with a ratio of 46 polyploids: 52 diploids. He observed that polyploids (in cases of sympatry) tend to occupy the margins of the distribution areas. According to Edwards and Bohlen (1996), the association between parthenogenesis and high polyploidy in earthworms produces an unexpected level of heterozygosity, an advantageous condition that provides resistance to environmental stress.

5.2 Sexual Selection in Cross-Fertilization Earthworms

In simultaneous hermaphrodites, a trade-off between male and female sexual functions is expected because the two sexes share limited resources from the same individual. In addition, the strategy that maximizes fitness is different for the male and female functions. This has been explained previously by Bateman (1948), who showed that the higher the number of partners, the higher the fitness for the male function because it produces small sperm cells. Nevertheless, female function maximizes its fitness by seeking high quality mates because it produces large eggs and this function has to invest in cocoon production. As a consequence, there is a conflict between the sexes. Indeed, Porto et al. (2008) found a negative relationship between the present investment in male function and the future fertility of the female function in their research on *E. andrei*. Sexual selection is expected to occur because of female function as long as a sufficient number of mates are available.

5.2.1 Precopulatory Sexual Selection

Copulation is very costly and involves sperm and mucus production and long periods of time. Consequently, precopulatory selection is expected in environments where the density of earthworms is high.

One of the factors that could influence precopulatory sexual selection is the female fecundity of the partner, which may be related to body size. Large earthworms have not been found to produce more cocoons (Tato et al. 2006; Butt and Nuutinen 1998) but they do tend to produce heavier cocoons and larger offspring (Michiels et al. 2001). Size-assortative mating was indeed observed in the field for the epigeic *E. fetida* (Monroy et al. 2005) and for the endogeic *H. elisae* (Novo et al. in press), as well as in laboratory experiments for the anecic *L. terrestris* (Michiels et al. 2001). Earthworms selected similar-sized partners. Because every earthworm seeks a bigger partner, equilibrium is finally reached, resulting in partners with a similar weight, thus balancing the expectations of both mates on female and male functions. In the particular case of epigeic and anecic worms, which can copulate at the surface, this general tendency could be reinforced by a trade-off; worms can either select a bigger, more fecund partner or a smaller partner, which would decrease the risk of predation.

In ongoing laboratory experiments with *H. elisae*, we have observed that there is no such size selection in virgin individuals, although the bigger virgin individuals always managed to copulate so they seem to be more desirable.

Aside from size, reciprocation is sought from a potential partner. In simultaneous hermaphrodites, the primary purpose of mating is to fertilize the eggs of their partners, rather than to fertilize their own eggs. Therefore, the conflict of two earthworms copulating would be the amount of sperm that each of them is allowed to give (Michiels 1998).

Finally, the quality of the place where cocoons are deposited after copulation and the suitability of the burrow for offspring development (i.e., the moisture or litter content) could be important factors for precopulatory assessment. Ortiz-Ceballos and Fragoso (2006) studied parental care in *Pontoscolex corethrurus* and *Balanteodrilus pearsei*. They found that both species build up a chamber that they periodically clean and surround with fresh casts where a single cocoon is deposited. Grigoropoulou et al. (2008) observed that *L. terrestris* deposits the cocoons inside burrows, which may offer a protective location from the physical environment or may represent parental investment as they were also found to be coated with earthworm casts. These casts could be a means of maintaining the moisture content or protecting cocoons from predators.

The mechanism through which earthworms choose a mate, assess size, test reciprocity, or assess the burrow quality of their potential partners remains unknown, although there are some data on these factors. Chemical cues have been suggested in earthworms as a mechanism of finding and attracting the mate (Olive and Clark 1978; Edwards and Bohlen 1996).

Grove and Cowley (1926) suggested the existence of a courtship in *E. fetida* as they observed short and repeated touches between partners before mating. This type of contact, executed with the prostomium, was also observed by Nuutinen and Butt (1997) in *L. terrestris* and could last 90 min. The prostomium has been described as a sensory lobe with many chemoreceptors or sensory cells (Wallwork 1983).

During contact, the clitellum and associated structures could be indicators of female functionality and glandular margins of the male pores could be indicators

of male functionality. These structures could provide a means of evaluating the partner and assuring reciprocation. Reciprocation can also be assured by increasing the copulation time, which would prevent the partner from copulating with other earthworms. In addition, Nuutinen and Butt (1997) observed that *L. terrestris* visited the potential mate's burrow by inserting its anterior segments, but retaining the posterior segments in their own burrows, as a mechanism to evaluate the quality.

In case of the size assessment, it is also suggested that assortative mating could be due to a physical incompatibility of the copula among individuals of different sizes (Michiels et al. 2001), although this incompatibility would only result from large differences in size.

These selective forces depend on other factors, such as the density of earthworms or the distance of potential mates. Indeed, the low dispersal ability of these animals provides a restriction in the number of available mates. Earthworms have low migration rates, with observed natural dispersal rates of only 1.4–9 m year^{-1} (Ligthart and Peek 1997; Hale et al. 2005) and are therefore expected to mate with partners living in their vicinity. In addition, in the case of the earthworms who copulate at the surface, a smaller distance to the partner would also minimize the risk of predation. There is evidence for this selective limitation produced by distance. Nuutinen and Butt (1997) investigated burrow visit patterns in *L. terrestris* and found that the nearer the burrow opening was, the more visits the worms made to assess the potential partner quality. In addition, Sahm et al. (2009) showed mate choice in the same species for its closest partner and Novo et al. (in press) found that *H. elisae* do not move long distances to find mating partners. Nevertheless, this low dispersal could cause inbreeding, which is generally accepted to be unadaptative and would reduce the fitness of the offspring. Partner selection has not been found to be dependent on relatedness (i.e., kin recognition), and Novo et al. (in press) did not find a correlation between mating probabilities and the level of heterozygosity in *H. elisae*. Regarding this, differential investment in offspring is thought to occur (Velando et al. 2006, see Sect. 8.2.2).

Finally, parasite concentrations may influence mate choice, since they can have a negative effect on earthworm growth as shown by Field and Michiels (2005) for the association between *Monocystis* and *L. terrestris*. In addition, earthworm skin color could be positively correlated with parasite concentration (Field et al. 2003), which could be a sign used to evaluate partners. Nevertheless, Sahm et al. (2009) failed in an attempt to show a relationship between parasite concentration and mate choice, and more studies are needed to assess this correlation.

5.2.2 Postcopulatory Sexual Selection

In spite of the precopulatory sexual selection, multiple mating is common in earthworms (Monroy et al. 2003; Sahm et al. 2009; Novo et al. in press) and all the allosperm received is stored and sometimes mixed (Novo et al. in press) in the

spermathecae. Therefore, postcopulatory sexual selection such as sperm competition (Parker 1970) or cryptic female choice (Thornhill 1983) could be expected.

The sperm remains viable in the spermathecae until fertilization. Butt and Nuutinen (1998) observed that *L. terrestris* was capable of successfully maintaining the received sperm up to 6 months. Meyer and Bowman (1994) reported that *E. fetida* continued cocoon production for up to 12 months after the earthworms were isolated from their partner, although these authors did not measure viability. Garvín et al. (2003) reported spermathecae full of spermatozoa during diapause in *H. elisae*. This would be advantageous for species with poor dispersal capacities or for species that occur in low densities that can copulate at any time of the year.

The maintenance of sperm for such a long time implies the existence of some kind of preservation mechanism. There is evidence suggesting that the spermathecal epithelium actively contributes to the successful maintenance of sperm by providing a favorable luminal environment (Grove 1925; Varuta and More 1972) or by producing nourishing substances (Vyas and Dev 1972; Jamieson 1992; Novo et al. (unpublished data))

A possible mechanism for postcopulatory sexual selection developed by the recipient is sperm digestion. Richards and Fleming (1982) observed spermatozoal phagocytosis by the spermathecae of the facultative parthenogenetic *Dendrobaena subrubicunda* and other lumbricids. This is likely related to the removal of aging or aberrant sperm during the months when cocoon production was minimal. Novo et al. (unpublished data) found sperm degeneration in the central area of spermathecae from *H. elisae* (Fig. 5.1a, b). These authors also observed sperm intrusions into the epithelium of spermathecae with high sperm contents, although these intrusions seemed to occur in areas where the sperm sought more nutrients rather than phagocytosis processes (Fig. 5.1c). Future ultrastructure studies will shed light on these mechanisms.

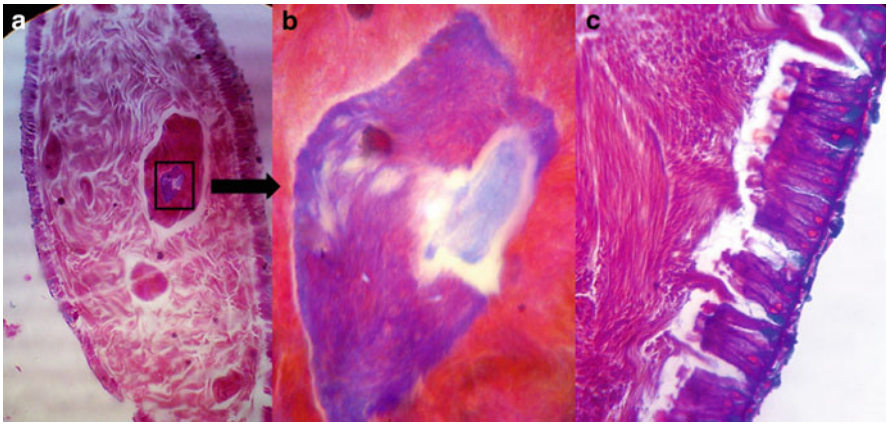

Fig. 5.1 Histological preparations of the spermathecae from *H. elisae*. Sperm degeneration (**a** and **b** in detail). Sperm intrusions in the epithelium of the spermathecae (**c**)

Another strategy for cryptic female choice could be the differential storage of the received allosperm within the spermathecae. The recipient can control the storage of sperm by increasing the complexity of these organs. Different species of earthworms have different numbers of spermathecae (Sims and Gerard 1999), although Novo et al. (in press) demonstrated using microsatellite markers that the four spermathecae from *H. elisae* contained sperm from the same individuals. Grove and Cowley (1926) observed that the transmission of sperm in *E. fetida* typically occurs on both sides of the individual, whereas in *L. terrestris* some individuals were found to have spermatophores on only one side of their body (Butt and Nuutinen 1998).

Moreover, some earthworms present different sperm loads within a single spermathecae. This has been observed in some hormogastrids (Qiu and Bouché 1998), and in *Megascolides australis*, in which spermatozeugmata (i.e., sperm in orientated bundles) were reported by Van Praagh (1995). In addition, the spermathecae may include one or more diverticula that arise from the duct (Butt and Nuutinen 1998).

Finally, the amount of sperm stored in each spermatheca could be controlled, and this occurs for *L. terrestris*, which predominantly store sperm in the two posterior spermathecae when there is no injection of allohormones (Koene et al. 2005, see later). Garvín et al. (2003) also observed that the second pair of spermathecae seems to be the main recipient of spermatozoa in *H. elisae*. However, Velando et al. (2008) showed that *E. andrei* distributes the sperm equally among the four spermathecae.

Cryptic female choice may also be achieved through differential investment in offspring. Velando et al. (2006) found that *E. andrei* adjusted the breeding effort to the degree of mate relatedness, showing that inbreeding and outbreeding cause a strong reduction of cocoon production, especially in genetic lines with high reproductive rates.

Sexual selection drives the evolution of strategies that increase the chances of fertilization for the donated sperm as a means of increasing paternity. Some of these strategies have been observed in earthworms. Velando et al. (2008) reported a behavior that could promote sperm competition in *E. andrei*, which can have a high degree of control over their own ejaculate volume after evaluating their partners. This species donated three times as much sperm as they did normally when mating with a nonvirgin mate. Moreover, such increases were greater when the worms mated with larger partners. Mariño et al. (2006) also showed a sperm trade in *E. andrei*, which adjusted the amount of sperm they release to the volume they receive from their mating partner during copulation. In addition, the total sperm volume they found in the spermathecae was correlated to the recipient's body mass, indicating that this adjustment is in accordance with the quality of the partner.

Koene et al. (2002) proposed that during mating, *L. terrestris* use their copulatory setae to pierce their partner's skin to inject an allohormone produced by the setal glands which manipulates the reproductive physiology of the partner and damages the body wall. The injection of this substance provokes a higher uptake of sperm, a more equal sperm distribution over the four spermathecae, and an increase the amount of time occurring before the next mating. The damage caused

by the injection itself could incur a considerable cost that inhibits another mating (Koene et al. 2005).

5.3 Parthenogenesis

5.3.1 Definition

Parthenogenesis is a very wide collective concept. Historically, classical authors addressed this concept on several occasions; although not defining the concept or providing an experimental approach, authors posed hints regarding the existence of this kind of reproduction. Although Bonnet provided experimental proof for this kind of reproduction in aphids in 1762, it was not until 1849 that Richard Owen coined the term. He defined parthenogenesis as "procreation without the immediate influence of a male". As this general concept could include several typically asexual modes of reproduction such as fission or budding, several authors attempted to create new definitions for this term. A century later, Suomalainen (1950) defined it as "the development of the egg cell into a new individual without fertilization". Later, Beatty (1957) defined it first as "the production of an embryo from a female gamete without the concurrence of a male gamete, and with or without eventual development into an adult", but modified the definition in 1967 (Beatty 1967) by substituting "without any genetic contribution from a male gamete" for "concurrence of a male gamete". In this way, Beatty extended the definition to include special types of parthenogenesis such as gynogenesis. Nevertheless, all of these definitions give rise to some terminological difficulties.

5.3.2 Types of Parthenogenesis in Earthworms

Several classifications have been used to define the different types of parthenogenetic mechanisms. To understand earthworm classification of parthenogenesis, it is worth mentioning the classifications proposed by Thomsen (1927); Ankel (1927); Suomalainen (1950) and White (1973); these are mainly based on the mode of reproduction, sex determination, and cytology.

The system of classification proposed by Thomsen (1927) and Ankel (1927) points out two main points: the zygoid–azygoid status of an individual and the maintenance of the zygoid chromosome number. It includes two main categories: generative or haploid parthenogenesis (in which chromosome reduction takes place in the eggs, and consequently the parthenogenetic offspring have an azygoid – haploid-number of chromosomes), and somatic parthenogenesis, in which parthenogenetic offspring have a zygoid–diploid or polyploid-chromosome number.

The difference between the two categories basically depends on the absence (apomixis) or presence (automixis) of chromosome conjugation and reduction. Both concepts are synonymous with White's concepts of ameiotic and meiotic parthenogenesis, respectively.

When considering sex determination, it is especially useful to use the classification of parthenogenesis proposed by Suomalainen et al. (1987): arrhenotoky, thelytoky and deuterotoky, or amphitoky (unfertilized eggs producing only male descendants, only females, or descendants of both sexes, respectively).

Parthenogenetic earthworms are generally automictic and thelytokous. Following the cytological studies of Muldal (1952); Omodeo (1951, 1952, among others) and Casellato and Rodighiero (1972), there is a premeiotic doubling of the chromosome number at the last oogonial divisions resulting in endomitosis, followed by the formation of chiasmatic bivalents and regular meiosis with the extrusion of two polar bodies. The genetic consequences of this cytological mechanism are similar to those of apomixis (i.e., the formation of clonal animals), as synapsis is restricted to sister chromosomes that are exact molecular copies of one another. The immediate genetic consequence of this mechanism is that heterozygosity is maintained. Following White (1973), all bivalents are structurally homozygous and multivalents are never formed. Consequently, this kind of reproduction is perfectly compatible with different degrees of polyploidy, especially in odd-numbered levels (Fig. 5.2).

Only one exception to the parthenogenetic mechanism described above has been found. *Dendrobaena octaedra* shows apomictic parthenogenesis: the chromosome number is not doubled in the oogonia, the chromosome number of the oocytes is unreduced, and there is only one equational maturation division (Suomalainen et al. 1987). For this species, Omodeo (1953) and later Gates (1972; as explained later in this chapter) described different parthenogenetic forms with a huge degree of morphological variation, which makes it very difficult to establish the evolutionary relationships among them. Omodeo (1953) suggested that "it could be the result of a breakdown of developmental canalisation in the absence of stabilizing selection", while White (1973) indicated that "it seems more likely that it indicates the coexistence of numerous biotypes differing significantly from one another genetically, even if not in their visible cytology".

Parthenogenesis is one of the main sources of morphological variability within reproductive structures of earthworms. This variability is related to the reduction in the investment in male structures: seminal vesicles, testes, spermathecae, genital setae, and prostates are reduced or even lacking; there is no sperm production (i.e., lack of iridescence in male funnels and spermathecae); and spermatophores are lacking (in some cases they are produced but are invariably empty). In *Octolasion tyrtaeum* (Muldal 1952; Jaenicke and Selander 1979), male structures are not reduced and pseudogamy is shown: individuals copulate to exchange spermatophores that are invariably empty. Thus, although spermatozoids are not necessary, this species needs a mechanical or chemical stimulus to trigger reproduction. Polymorphic degradation of reproductive structures is often observed in parthenogenetic organisms. In some species, such as *Eiseniella tetraedra* even hypergynous

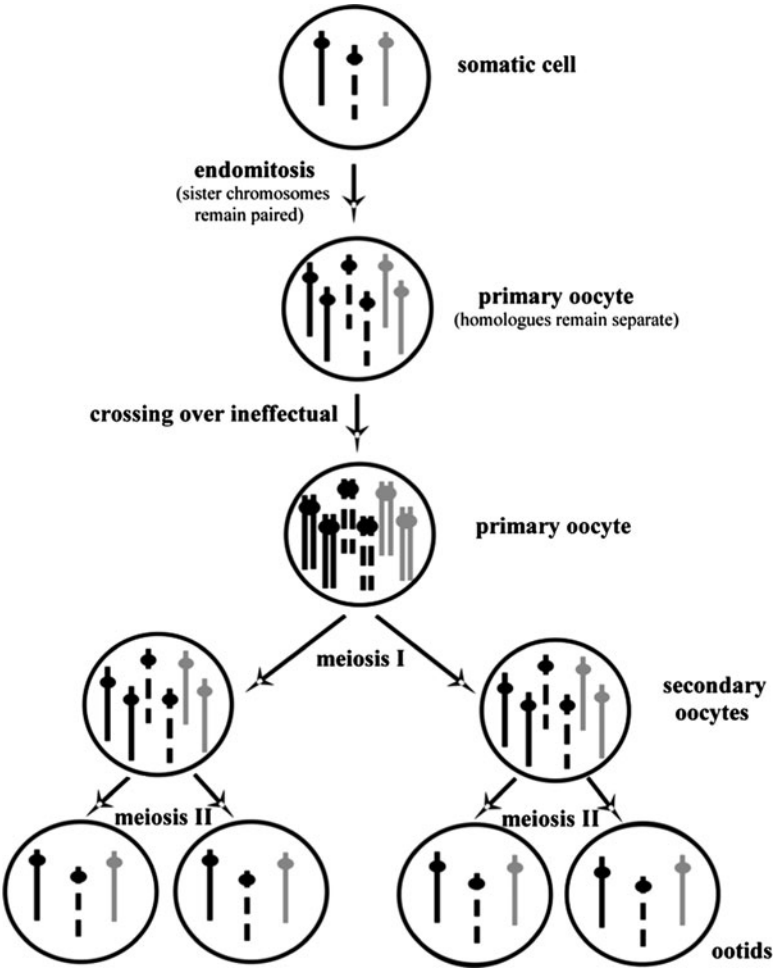

Fig. 5.2 Automictic parthenogenesis: the genetic consequences of premeiotic restitution

individuals (with an extra pair of ovaries) can be found (Jaenicke and Selander 1979). However, in other parthenogenetic earthworms such as *Aporrectodea trapezoides*, both primary and secondary male sexual characters, such as perithecal papillae, tubercula pubertatis, spermathecae, swollen male porophores, and seminal vesicles, are retained. Recent studies show that pseudogamy is not observed in this species (Fernández et al. 2010). As discussed later, this seems to suggest very different origins of parthenogenesis in the different species.

Parthenogenesis is not homogenously distributed in earthworms; it is only found in lumbricids and megascolecids. It is curious that it is not found (or not known to occur) in glossoscolecids or hormogastrids; this clearly shows that their life traits or evolutionary histories should be completely different and that somehow parthenogenesis and even polyploidy are not compatible or viable in this family.

5.3.3 Parthenogenesis and Polyploidy

Most part of the parthenogenetic earthworms are polyploids. Polyploidy ranges from tri- to dodecaploidy. From a cytogenetical point of view, automictic biotypes should be diploid (White 1973); nevertheless, in parthenogenetic lumbricids, polyploidy is the most common phenomenon. This is because, as explained later, the automictic mechanism in most lumbricids is premeiotic doubling, which leads to genetic consequences similar to an apomictic mechanism, leaving levels of heterozygosity unchanged from generation to generation (Suomalainen 1950). Because of premeiotic doubling, no multivalents are formed, so pairing only occurs between genetically identical sister chromosomes; this mechanism is compatible with odd-numbered polyploidy, as only bivalents are formed. This is the complicated chromosomal background that can give rise to different ploidy levels even within the same species. For example, in *Dendrobaena rubida*, diploid, triploid, tetraploid, hexaploid, and octoploid biotypes are known to occur, which clearly shows the extremely high liability of the genetic system. It has been proposed that automixis could be a step before apomixis (White 1973), which could mean that most lumbricids could be evolving toward an apomictic parthenogenesis. Polyploidy could be common in earthworms, as animals lacking the chromosomal determination of sex are particularly prone to this kind of reproduction, which is the main mechanism preventing the establishment of polyploidy in animals (White 1973). One of the main advantages of polyploidy in parthenogenetic species is the increase in genetic variability.

Since no study to date has elucidated the origin of parthenogenetic earthworms (as explained later in this chapter), it is not known if parthenogenetic earthworms may have arisen from hybridisation processes. These kinds of processes have been found to be very common mechanisms causing asexuality (only to the extent that parthenogenesis can be considered to be asexual reproduction) in animals and plants (Delmotte et al. 2003). Following this assumption, polyploidy (and particularly allopolyploidy) could provide important advantages, such as heterosis, to parthenogenetic species. This strong advantage could lead the parthenogenetic morphs to have more general purpose genotypes, allowing them to adapt to a wider range of environmental conditions than their sexual amphimictic ancestors (White 1973). There is much evidence that hybrid vigor could be responsible for the success of polyploids, but there is insufficient information to determine this with certainty.

5.3.4 Genetic and Ecological Consequences of Cloning

As stated by Hughes (1989), it is extremely difficult to define the advantages or disadvantages of parthenogenesis, as these depend on the situation; for some groups of animals, parthenogenesis is tremendously advantageous, while in others it is not.

Therefore, natural selection should control the pattern of occurrence in each group of animals.

Using molecular tools, very different degrees of genetic variability have been reported in different species. Both with allozyme electrophoresis and with mitochondrial gene sequencing, genetic variability was recorded as being high in *D. octaedra* (Haimi et al. 2007; Terhivuo and Saura 1996; Cameron et al. 2008) and *Aporrectodea rosea* (Terhivuo and Saura 1993; King et al. 2008), but low in *O. tyrtaeum* (Jaenicke et al. 1980; Heethoff et al. 2004) and *O. cyaneum* (Terhivuo and Saura 2003). In *A. trapezoides*, both mitochondrial and nuclear sequences resulted in an extremely high number of clones (Fernández et al. unpublished data.).

Judging from the number and distribution of parthenogenetic earthworms, one could expect that parthenogenesis is quite advantageous in this group. Parthenogenetic earthworms are widespread and very abundant, especially among peregrine species (Blakemore 1994) such as *A. rosea*, *A. trapezoides*, or *O. tyrtaeum*. Hughes (1989) pointed out the following advantages of parthenogenesis: both high levels of heterozygosity and exceptionally fit genomes, which are maintained and inherited by avoiding recombination and segregation; high reproductive rates, which could potentially be doubled by avoiding the production of males (i.e., no twofold cost in parthenogenetic reproduction); high colonizing abilities, since there is no need to mate; high values of reproductive potential, enabling clones to quickly replace losses; advanced polymorphism generated from selection at the level of the genome; and the delay or prevention of senescence as somatic replicas from undifferentiated somatic cells are generated. In reference to the last advantage, Hughes (1989) pointed out that several clones of oligochaetes did not show any signs of senescence after having been maintained for many generations.

5.3.5 *The Species Concept in Parthenogenetic Earthworms*

Parthenogenetic earthworms were wisely defined as "systematist's nightmares" by Blakemore (1999). The biological species criterion cannot be applied to parthenogenetic earthworms, as each individual meets the criterion of being completely reproductively isolated not only from the parental species, but also from every sister clone. Several authors have attempted to resolve this problem, but an agreement has never been reached. Mayr (1963) suggested that the best solution would be to use a morphological criterion. Following this author (1963), a parthenogenetic species would be the one that "results in the combination of a single species of those asexual individuals that display no greater morphological difference from each other than from conspecific individuals or populations in related bisexual species". He also proposed that clones can be combined into collective species when no essential morphological or biological differences have been observed. To complete this criterion, the author also argued that if a parthenogenetic line originated from an amphimictic species by an irreversible chromosomal event (such as polyploidy), it should be considered to be a separate and sibling species, although almost no

morphological differences could exist. This criterion has traditionally been used to define species in parthenogenetic lumbricids, though it can be difficult to apply as the degree of morphological variation is sometimes slight and the features defining parthenogenetic and even amphimictic species can overlap. This is a particularly big problem in complexes of very similar species containing both amphimictic and parthenogenetic species such as the "*Aporrectodea caliginosa* species" complex. In this context, other approaches, as discussed later, could be essential not only for properly defining parthenogenetic species, but also for determining the taxonomic status of each form in these species complexes.

Following Gates (1974), "the species is understood to include not only the interbreeding population, but also all recently evolved uniparental strains, clones, or morphs that clearly are affiliated with it". This statement is useful when intermediate forms are found, but still does not solve the problem of how to resolve the status of parthenogenetic species with unknown (or extinct) amphimictic parental species. Another option would be to use the phylogenetic concept of species based on molecular markers, which would provide information about the genetic divergence between morphs or species. However, these tools are not so well developed in earthworms that they could provide a good idea as to the exact amount of divergence that should be used to differentiate between species. In addition, there is evidence of different degrees of divergence among closely related species in the different earthworms groups. The best way to define a parthenogenetic species (and amphimictic species, particularly when dealing with complex of species) is to use an integrative concept of species, using ecological, behavioral, morphological, and molecular data. A species should not be given a name if its biology is not well understood, but then, it is completely necessary to name the species. Parthenogenetic species are very common among the earthworms, and thus a solution needs to be found. The ideal study would be one using all of the available approaches to examine the same individuals so as not to incorporate any source of error or introduce any possible mistakes when identifying species. Making comparisons with previously published data is dangerous because different authors might have incorrectly identified species when dealing with parthenogenetic morphs or species from a complex, in which intermediate forms are typically found. The best means of eliminating this uncertainty is to deposit the individuals used in the experiments into a collection.

Gates (1974) categorized parthenogenetic morphs of *D. octaedra* using the presence or absence of different reproductive male structures. Gates (1974) defined morphs lacking spermathecae, male terminalia, testes, testis sacs, or seminal vesicles or those lacking several of these structures (e.g., athecal anarsenosomphic, with or without testes). He also included two categories of intermediate morphs with an incomplete or asymmetrical deletion of the above organs: hermaphroditic parthenogenetic morphs were defined as those that had reproductive organs in a juvenile state, while hermaphroditic morphs used biparental reproduction and were also parthenogenetic. Unfortunately, few studies have demonstrated the existence of these forms in every parthenogenetic species; the knowledge about the extension and degree of parthenogenetic morphs in parthenogenetic species is quite limited.

This is a problem both for clarifying the taxonomy of earthworms using this type of reproduction, and for understanding the origin of parthenogenesis in these species.

Gates (1974) and Blakemore (1999) suggested that parthenogenetic morphs should be given a name only when the parental amphimictic species can be determined. We totally agree with this statement. Nevertheless, as Blakemore suggested, the origin of the name, regardless of whether it was based on morphs or parthenogenetic forms, has no effect on the availability of a taxonomic name (ICZN 1999, Article 17.3). Moreover, Gates (1972) suggested that provision of names for all intermediate morphs of such species complexes was *ridiculous*.

Another limitation, as stated by Suomalainen et al. (1987), is that there are still very few examples of taxonomic diversification beyond the species level in parthenogenetic earthworms.

5.3.6 The Origin of Parthenogenetic Forms

Amphimictic ancestors of parthenogenetic forms are well known in many different animal groups, but this is not the case for Lumbricids. Hybridization has been proposed several times (e.g., Suomalainen et al. 1987) as a common origin of parthenogenetic animal species such as fishes, lizards, and salamanders. Among invertebrates, there are many examples of parthenogenetic forms originating from Hybridization in the literature. This is the case, for example, for parthenogenetic forms in delphacid leaf-hoppers or stick insects belonging to the genus *Acanthoxyla* which were described as having two haploid genomes, one of which came from an amphimictic parental species (Buckley et al. 2008). Suomalainen et al. (1987) also gave some examples among invertebrates in which parthenogenesis seems to have arisen through a single mutational event, or through multiple events. In these cases, parthenogenesis was a polyphyletic condition within a single species as, for example, in the psychid moth *Solenobia triquetrella*.

Little is known about the origin of parthenogenetic earthworms. Molecular biology will be very useful in shedding light on this topic. Several tools can be useful in reaching this goal. Traditionally, some studies using allozymes have been used to check genetic variability in parthenogenetic and sexually reproducing species that are related, such as *A. trapezoides* and *A. caliginosa* (Cobolli Sbordoni et al. 1987). However, the information obtained using this technique was not sufficient to evaluate hypotheses regarding the origin of parthenogenetic forms. An appropriate first approach would be to compare phylogenies using both mitochondrial and nuclear genes. To determine whether parthenogenetic species originated from hybridisation, alleles could be cloned in nuclear genes to check for the presence of different haploid genomes in diploid and, especially, polyploid parthenogenetic earthworms.

As stated earlier, there is a strong variation among parthenogenetic earthworms regarding the type of parthenogenesis that is observed; most of the species are automictic, but at least one is apomictic. Similarly, some species are pseudogamic

while others are not; some lack spermathecae while others have an extra pair of ovaries. The fact that parthenogenetic mechanisms are very labile in earthworms provides strong evidence that parthenogens could have originated in a number of different ways. Molecular biology will allow us to better understand why parthenogenetic earthworms have been so successful.

5.4 Conclusion

Reproduction models in earthworms are much more variable than it could seem *a priori*. Although direct cross-fertilization hermaphroditism may be seen as the most usual model, it is common to find different ones as self-fertilization or parthenogenesis. Even within the most widespread strategy, it is possible to find variations, such as presence of spermatophores.

During the last years, a great research effort has been made to shed light on some aspects of sexual selection, such as mate assessment, copulatory behavior, and sperm competition. Nevertheless, very interesting processes as origin and maintenance of parthenogenesis in earthworms are mainly unknown. Deeper research on both aspects would allow us to better understand the reproductive biology of these animals.

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sciencealert

ENVIRONMENT

Here's Why Virgin Birth Is Scientifically Possible

JENNY GRAVES 24 DEC 2015

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Christmas seems an appropriate time to ask whether it's biologically possible to have a virgin birth. And you may be surprised to hear that it is possible – just not for humans, or any other mammals. Experiments with mice and other mammals show an egg must be fertilised with a sperm to kick off development of any kind. Just stimulating a mammal egg with chemicals or electricity doesn't trigger it to divide normally.

It seems you need particular proteins from sperm to set up waves of calcium ions in the egg, which trigger further changes leading up to copying all the DNA and chromosomes, and dividing into two cells.

But you need more than just a protein trigger supplied by the sperm. You also need two copies of each chromosome in the fertilised egg. Normally one set is provided by the mother (in the egg nucleus) and one by the father (in the sperm nucleus).

You can engineer a mouse egg to have both nuclei from the same sex, but this doesn't work. An egg with two maternal nuclei goes some of the way to making an embryo, but it shrivels up because there is little development of placenta. If both nuclei come from a male there is the opposite problem: a lot of placenta but hardly any embryonic development.

But why not?

It turns out that there are more than 30 imprinted genes that are active only if they come from a father through sperm. There are another 30 plus that are active only if they come from the mother. So genomic imprinting prevents virgin birth in all mammals, including humans.

Genomic imprinting is the different activity of genes according to which parent they come from. It was discovered in the 1990s, with its mechanism only sorted out in the last decade. It seems that the inactive gene is not mutated, but is silenced by attaching chemical groups to the DNA. These chemical groups are put onto genes in the testis or the ovary, and are removed during growth of the embryo.

Evidence that this silencing messes up uniparental embryos comes from engineering mice in which the imprinting process on one key gene is disrupted, leading to viable embryos with two mothers.

But it's still a mystery as to why imprinting evolved. Was it selected for because it prevented virgin birth? Or was it the result of a war between the mother's and father's genes? This 'sexual antagonism' is suggested by the functions of many of the imprinted genes.

Generally, active genes from the father directly or indirectly promote growth, whereas active genes from the mother suppress growth. It has been suggested that the father's genetic interests are best served by producing the biggest, toughest baby, whatever the cost to the mother (you can always find another female to mate with). The mother's genetic interests are best served by limiting the claims on her health and energy so she can survive to bear more children.

Virgin birth in other animals

But virgin birth *is* possible, if you're a reptile or a fish. For instance, pythons and Komodo dragon females that were long isolated were found to produce young that had only genes from the mother. It now seems to be an option in some snake species, and is known in several species of shark. Handy when there are no males around!

In fact, there are several lizards that are exclusively female. Some whiptail and gecko species in the arid southwest of the USA and the hot and dry interior of Australia have females whose unfertilised eggs develop fully – all into daughters.

The process is called parthenogenesis (literally 'virgin creation'). The animals that practise it (snakes, sharks and lizards) don't have to worry about genomic imprinting, which does not occur in egg-laying animals.

There are several ways reptiles can accomplish this. A female can make fertile eggs with the right number of chromosomes either by fusing an egg cell with another cell with one set of chromosomes. Alternatively the egg progenitor can undergo a variant form of division that leaves two copies of the genome.

This isn't exactly cloning, because the mother's gene copies are scrambled, but it does mean that all the genes of the offspring come from the mother.

Why sex?

The occurrence of parthenogenesis in reptiles poses a puzzle: what is the point of sex anyway? Wouldn't your genes do better in the evolutionary race if your offspring received genes only from you? This 'twofold cost of sex' has been a serious question in the field for 80 years.

The answer seems to be that although parthenogenesis works fine in the short term, it will always lose out in the long run because recombining two genes each generation is a great way of scrambling the combinations of proteins that pathogens see.

A pathogen that can infect one individual can also infect others with the same genes, so it's no point in having many cloned copies. For

instance, the female-only Australian gecko is very susceptible to mite infestation.

So the answer to the question of whether virgin birth is a real possibility is: yes, unless you are a mammal.

Jenny Graves, Distinguished Professor of Genetics, La Trobe University

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Parthenogenesis and Natural Clones

Robert C. Vrijenhoek

Rutgers *University*

GLOSSARY

amphimixis Sexual reproduction; the mixing of the genes from two distinct individuals; involving the recombinational effects of meiotic reduction and fusion of gametes.

apomixis Asexual reproduction without chromosome reduction or fusion of gametes; ameiotic parthenogenesis; retains parental heterozygosity.

automixis Asexual reproduction with chromosomal reduction but without fusion of gametes; meiotic parthenogenesis; rapidly leads to complete homozygosity.

endoduplication Duplication of the entire chromosomal set without cell division prior to meiosis.

gynogenesis Sperm-dependent parthenogenesis; sperm are used to activate embryogenesis but fusion of egg and sperm nuclei does not occur; pseudogamy.

hemiclone A haploid clonal genome that is transmitted without recombination by hybridogenetic females.

hybridogenesis The perpetuation of a hybrid genotype (AB) by hemiclonal inheritance in which the maternal genome (A) is transmitted to eggs; the paternal genome (B) is discarded during oogenesis and restored by true fertilization with sperm from males of a sexual host species B.

pseudo gamy Sperm-dependent parthenogenesis in plants; pollen is required to activate seed development, but the seed nucleus is produced clonally.

tychoparthenogenesis Occasional or accidental parthenogenetic development in unfertilized eggs.

Parthenogenesis (virgin birth) is reproduction via eggs but without sex. Eggs develop into new individuals without fertilization by sperm. Parthenogenetic lineages occur in many plant and animal taxa, and they may flourish under a variety of ecological conditions. Nevertheless, individual clones are believed to be evolutionary dead ends, because they lack the ability to respond genetically to changes in their physical and biotic environments.

I. PARTHENOGENESIS AND ASEXUAL REPRODUCTION

Reproduction does not require sex, or amphimixis, a complex process that involves two basic elements: (i) meiotic reduction-chromosomal segregation, assortment, and crossing over that generate an immense variety of haploid gametes; and (ii) syngamy-fusion of gametes that produces unique new individuals in each generation. Mixing the genotypes from different individuals (recombination) is the essential characteristic of sex in eukaryotic organisms, and circumvention of these processes leads to parthenogenesis and cloning.

Vegetative reproduction (budding, fragmentation, fission, etc.) is common in plants and some invertebrate animals. Although comparable to parthenogenesis in producing clones, vegetative modes of reproduction should be distinguished because they do not involve egg production and meiotic processing of chromosomes. Chromosome processing may be necessary to reset imprinted DNA methylation patterns and restore developmental totipotency in some organisms. Additionally, fertilized seeds and eggs (and subsequent larvae) are often the essential dispersal phase of many plants and animals. In most cases, vegetative propagules tend to remain close to the parent organism. Corals ordinarily reproduce by budding, but they employ sexual reproduction to

produce planula larvae, the dispersal phase of the life cycle. In an ecological sense, vegetative reproduction is more appropriately compared with growth than reproduction.

Cyclical parthenogenesis alternates between sexual and asexual egg production. Because cyclical parthenogens engage in periodic recombination, they are facultatively sexual. The cladoceran waterflea, *Daphnia pulex,* produces a new assemblage of clones after each cycle of sexual reproduction. Sexual reproduction generally is stimulated by high density or other forms of stress and is used to produce the overwintering eggs. However, some populations occurring at high latitudes and in more permanent bodies of water have given rise to obligately parthenogenetic lineages that no longer reproduce sexually.

True parthenogenesis is a strictly clonal form of reproduction that transmits the female's diploid (or polyploid) genome to eggs, which develop spontaneously into genetically identical daughters. The terminology favored by botanists is more precise in its distinction among cytological mechanisms involved in the production of eggs. The term apomixis (ameiotic parthenogenesis) is used to describe zygote production without chromosomal reduction (some researchers include vegetative reproduction under apomixis). Some apomicts eliminate the reductional division (meiosis I) and produce nonrecombinant eggs with a single equational division (meiosis II). Other ameiotic methods of egg production are known and the primary genetic consequences are strict clonal inheritance and retention of the maternal level of heterozygosity.

In contrast, automixis (meiotic parthenogenesis) restores diploidy by fusing various meiotic products. For example, some free-living *Rhabditis* nematodes fuse the second polar body with the egg nucleus. In most cases, automixis is comparable to self-fertilization and quickly leads to complete homozygosity. Some automicts produce normal haploid ova and then duplicate the generative nucleus in a subsequent mitotic division. Fusion of these mitotic products restores diploidy but leads to complete homozygosity in one step. Once automicts are completely homozygous, inheritance is effectively clonal.

Most parthenogenetic animals are functionally apomictic. They retain elements of meiosis while circumventing chromosomal recombination and reduction. For example, parthenogenetic whiptail lizards of the genus *Cnemidophorus* duplicate the entire chromosomal complement prior to meiosis, a process known as endoduplication. Because synapsis occurs between the duplicated pairs of chromosomes, meiotic recombination is genetically inconsequential. Eggs contain a functionally nonrecombinant version of the maternal genotype. A great variety of functionally apomictic mechanisms are known. Their common theme is the circumvention of reduction and recombination. Many parthenogenetic animals arose as hybrids, and functional apomixis effectively preserves their hybrid genotypes. Why functionally apomictic animals are more common than true apomicts is not understood. Perhaps, chromosomal processing during prophase of meiosis I is necessary for normal embryonic development.

Sperm-dependent modes of parthenogenetic reproduction also are known. Dandelions in North America (they were introduced from Europe) are pseudogamous apomicts: Pollination is necessary to activate development of endosperm tissue in the seed, but the generative nucleus develops apomictically. Pseudogamy is more commonly called gynogenesis in animals (Fig. 1). Despite the need for sperm, pseudogamous inheritance is strictly maternal and clonal. The fall cankerworm moth, *Alsophila pometaria,* has pseudogamous lineages that use sperm from males of a coexisting sexual lineage, but gynogenetic fish such as the Amazon molly, *Poecilia formosa,* use sperm from males of closely related sexual species. The need for sperm produces a kind of host-parasite relationship between sexually reproducing sperm donors and all-female gynogens. However, pseudogamous planarians are hermaphrodites, and they can use their own sperm. Although pseudogamous forms are not parthenogenetic in the strict sense (i.e., virgin birth), genetic consequences are the same: Syngamy does not occur and inheritance is clonal. Nevertheless, sperm-dependent versus sperm-independent forms of parthenogenesis function under very different ecological constraints.

Hybridogenesis, an unusual form of matrilineal inheritance that perpetuates a hybrid genotype, combines elements of parthenogenesis and sexual reproduction. The hybridogenetic fish *Poeciliopsis*

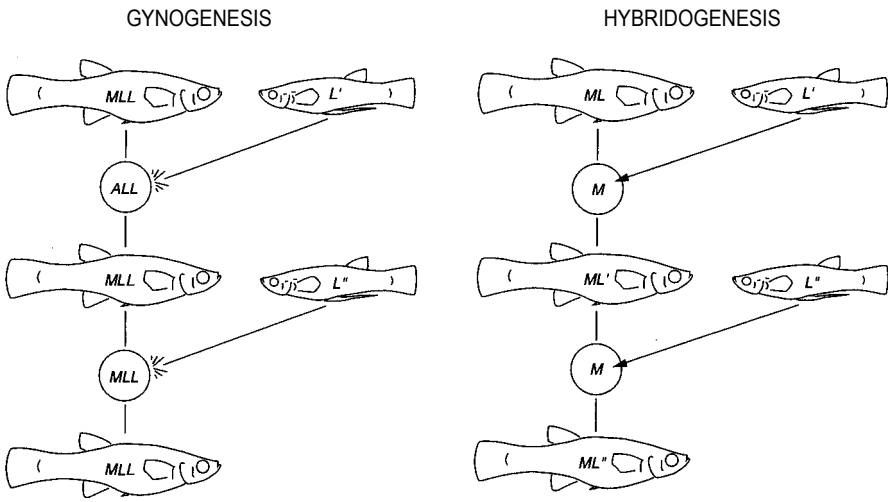

FIGURE 1 Gynogenetic and hybridogenetic reproduction in all-female fish (genus *Poeciliopsis) of* hybrid origin. The letters M and *L* represent whole chromosome sets from the sexually reproducing progenitors, P. *monacha* and P. *lucida.* The triploid gynogen, P. *monacha-2 lucida* (or *MLL),* has one set of monacha chromosomes and two sets of lucida chromosomes; and the diploid hybridogen, P. *monacha-lucida* (or *ML)* has one set of chromosomes from each species. Both the gynogen and hybridogen are pictured mating with males of P. *lucida.* During gynogenesis, the entire triploid genome, *MLL,* is transmitted between generations without recombination. Different markers associated with the sperm source *(L, L', L",* etc.) are not incorporated or expressed in the offspring. During hybridogenesis, only the haploid *M* genome (hemiclone) is transmitted to eggs. The paternal *L* genome is replaced in each generation.

monacha-lucida is a hybrid between the sexual species *P. monacha* and P. lucida. It is easier to describe hybridogenesis if we substitute the letters *M* and *L* for monacha and lucida chromosome sets of the hybrid (Fig. 1). Just before meiosis, these *ML* hybrids discard the *L* chromosomes. Functional eggs contain only a nonrecombinant *M* set that must fuse with sperm provided by P. lucida males, producing a new hybrid, *ML'.* New paternal genomes *(L, L', L",* etc.) are (i) drawn anew from the sexual gene pool in each generation, (ii) paired with the *M* genome, (iii) fully expressed in *ML* hybrids, and then (iv) discarded. The *M* genome is called a hemiclone because it comprises only half of the organism's chromosomal complement, and it is cloned. Populations of P. monacha-lucida usually contain several hemiclones, marked by distinct *M* genomes that were independently derived from P. monacha. The European water frog, Rana esculenta, also is hybridogenetic. Hybridogenesis is also found in some insects, but overall it is a rare form of clonal reproduction.

Numerous variations exist on these basic themes of clonal reproduction and parthenogenesis in plants and animals. The reference by Suomalainen and coworkers (1987) provide a useful summary of what is known about cytogenetic mechanisms.

II. ORIGINS OF PARTHENOGENS

Most plant and animal parthenogens (agamospecies or parthenoforms) have arisen relatively recently from sexual progenitors. Additionally, a large proportion of parthenogens are polyploids and many are interspecific hybrids. In the majority of cases, the sexual progenitors are extant and living sympatrically or parapatrically with the parthenogens (see Section III).

A. Spontaneous Origins

Meiotic parthenogens arise spontaneously in many plant and animal species. *Tychoparthenogenesis* (occasional development of unfertilized eggs) may be

favored in colonizing species that often find themselves at low density and without mates. Artificial selection can improve the rate of tychoparthenogenesis in *Drosophila mercatorum,* which suggests that automictic species such as *D. mangabierai* may have arisen spontaneously from tychoparthenogenetic ancestors. Nevertheless, the transition to automixis may be difficult if the sexual ancestors carry deleterious recessive mutations. Selection will rapidly eliminate automictic lineages that are homozygous for such mutations and fix the "lucky" lineages that lack them. Colonization, founder events, and small population sizes can purge the genetic load of the sexual progenitors and facilitate the transition to automixis.

B. Hybrid Origins

Many apomictic and functionally apomictic parthenogens arose as interspecific hybrids. All known asexual vertebrates are hybrids, as are many insects. The strong association between asexuality and hybrid origins led some researchers to suggest that cloning fixes heterosis (hybrid vigor) that may confer broad ecological tolerance. Although evidence exists for broad tolerance to physical stresses in some asexual plants, fish, and frogs, the phenomenon may be a consequence of interclonal selection for the best hybrid combinations rather than heterosis per se. Experimental studies with laboratory-synthesized hybridogenetic fish *(P. monacha-lucida;* Fig. 1) revealed that most hybrids were inferior to the parental forms; however, a small proportion of hybrid combinations had relatively high fitness. Fitness was not a consequence of heterosis; it was a consequence of the combining properties of parental genomes. Inferences about heterosis and fitness from comparative studies of natural parthenogens and their sexual counterparts are likely to be biased because we only see the successful genomic combinations in nature and not the failures that were purged by selection.

The association between parthenogenesis and hybridization may be a consequence of hybrid dysgenesis. Interspecific hybridization often leads to disruption of meiosis and sterility. Natural selection will preserve the lucky cytogenetic accidents that rescue egg production and restore or retain diploidy. Hybridization is one of a number of dysgenic processes that can produce windows of opportunity for the selection of ameiotic or functionally apomictic reproduction.

C. Parthenogenesis and Polyploidy

The majority of unisexual vertebrates, insects, and plants are polyploids. Although some researchers have suggested that elevated ploidy may produce superior genetic combinations, the association between polyploidy and parthenogenesis may also result from dysgenic processes. Accidental fertilization of a diploid (unreduced) egg will produce triploid progeny that typically are sterile. Such events create another window of opportunity for the selection of lucky cytological accidents that rescue egg production.

Prior establishment of functionally apomictic diploids can facilitate the elevation of ploidy because it removes the sterility barrier. For example, the triploid gynogenetic fish *P. monacha-2 lucida (3n =72;* Fig. 1) arose by addition of a second lucida genome *(in =24)* to a *P. monacha-lucida (2n =48)* hybrid. For most polyploids, we do not know whether unisexuality or polyploidy came first or if they arose together. If some of these polyploids outperform their diploid counterparts, enhanced performance may be a product of interclonal selection and fixation of the best genomic combinations from sexual ancestors rather than a direct consequence of elevated ploidy.

III. ECOLOGICAL CONSIDERATIONS

All other things being equal (i.e., survival, fecundity, niche requirements, etc.), an all-female lineage should rapidly replace its sexual relatives because a parthenogenetic female produces two daughters for every one produced by an equivalent sexual female. This twofold "cost of sex" may be exacerbated by numerous additional liabilities, such as the risks and energetic costs associated with finding a mate, courtship, and mating itself. Despite the costs of sex, asexual lineages generally have not completely replaced their sexual counterparts in animal taxa that regu-

larly produce clones. Williams (1975) referred to this ecological and evolutionary problem as the "paradox of sex." Why does biparental sexuality predominate so overwhelmingly despite its costs? Ecological studies that attempt to address this question have focused on the primary assumption behind this paradox- that all else is equal between sexual progenitors and derived asexual lineages.

A. Primary Fitness (Fecundity and Survival)

No investigator has succeeded in comparing the lifetime fertility and survival schedules of closely related sexual and asexual lineages in their natural environments, so it is impossible to say that everything else is equal with respect to primary fitness (fertility and survival). Some field and laboratory investigations have obtained data on components of fitness, although few generalizations can be drawn from the current studies. Gynogenetic and hybridogenetic Poeciliopsis have fecundities that are similar to those of their sexual counterparts. All-female reproduction is limited, however, by the availability of sperm from the sexual hosts. Parthenogenetic flies *(Drosophila)* and lizards *(Lacerta)* exhibit lower hatching rates than comparable sexual species. Finally, automictic lineages tend to have low hatching success, perhaps due to expression of deleterious recessive genes and inbreeding depression.

Survival differences have been observed in field and laboratory studies. Some unisexual fish *(Phoxinus eos-neogaeus)* and frogs (R. esculenta) may be more tolerant of thermal stresses than their sexual counterparts, but the differences do not appear to be generalizable. The roles of hybridity and selection for resistant clones are confounded in these organisms. Studies of survival under stress in Poeciliopsis revealed considerable variation among clones and no consistent advantage over the sexual counterparts for the various kinds of stress tested.

B. Geographical Parthenogenesis and General-Purpose Genotypes

Parthenogens should have superior colonizing abilities because they do not have to find mates when they initially occur at low density. Some researchers argue that parthenogens are general-purpose genotypes (jack-of-all-trades) that have wider ecological tolerances than their sexual counterparts. Other researchers argue that parthenogens are narrowly adapted fugitive species that escape from competition with their sexual ancestors. Biogeographical studies reveal that parthenogens are more frequent at the margins of a species range, at extreme latitudes, at higher altitudes, and in regularly disturbed communities-a pattern known as geographical parthenogenesis. It is unclear in most cases, however, whether this pattern is due to enhanced colonization abilities of parthenogens, an inability to compete with sexual progenitors in ecologically central areas, or an increased tolerance of ecologically marginal conditions.

Many widespread apomictic weeds appear to have general-purpose genotypes that can tolerate a wide range of environmental conditions. Selection in a varying environment should favor clones that fluctuate least in fitness. General-purpose clones may not be the best genotype in a particular set of circumstances but, more important, they avoid being the worst during many circumstances. Although the wide geographical distribution of many asexual plants and animals is often cited as supporting the general-purpose genotype hypothesis, such taxa may be composed of numerous cryptic (hidden) clones, each with different environmental tolerances, as found in some asexual waterfleas, brine shrimp, snails, and topminnows. Furthermore, a wide geographical distribution alone may not be sufficient evidence for general-purpose genotypes because a single widespread clone might occupy a narrow but universally available niche. For example, humans introduced dandelion (Taraxacum *offieinale)* clones to North America and their success is a consequence of human habitat disruption (grassy lawns).

The fugitive species aspect of geographical parthenogenesis does not apply to sperm-dependent parthenogens. Their colonization and competitive abilities are constrained by the need for sperm from coexisting sexual hosts. Outcompeting or geographically escaping the sexual host will lead to their own reproductive failure. Hybridogenetic and gynogenetic fish (Poeciliopsis) have relatively limited ranges

encompassed within the geographical limits of their sexual relatives and hosts, whereas some parthenogens, such as the cockroach *Pycnoscelus surinamensis,* have immense distributions, all outside the range of the putative sexual ancestors.

C. Niche Requirements

The niches of parthenogenetic clones and their sexual counterparts appear to differ in many cases. A sexual population should have greater niche breath than a single clone if the differences between genotypes contribute to a wider use of resources. For example, it is difficult to imagine a single jack-of-all-trades human clone (if humans were to be cloned) that has the breadth of talents of the entire human population from which it was drawn. The difference in niche breadth between a sexual population and a single clone will result in asymmetrical competition, in which the sexual lineage has a greater competitive impact on the clone than vice versa. However, an assemblage of ecologically divergent clones may equal or exceed the niche breadth of the sexual ancestors, leading to symmetrical competition and, perhaps, competitive exclusion of the sexuals.

Computer simulations of these ideas revealed that clonal invasion of the sexual niche proceeds from the margins to the center of the resource distribution. According to the frozen niche-variation model, a diverse array of clonal genotypes is frozen from the sexual gene pool. Interclonal selection will eliminate clones that overlap substantially with one another and the sexual ancestors and fix an assemblage of clones that maximally exploits the range of available resources. Sexual and clonal forms can coexist as long as competition remains asymmetrical and the combined niche of the clones is less than that of the sexuals.

Some hybrid parthenogens appear to occupy a weakly contested intermediate niche between the parental forms. However, hybrids are not necessarily intermediate for all niche-related characters. For example, some clones of the hybridogenetic fish P. *monacha-lucida* exhibit dominant phenotypes and extreme trophic behaviors. Hybridity does not necessarily constrain unisexual organisms to ecological intermediacy. Evidence also exists for niche separation between diploid and polyploid parthenogens in several taxa.

IV. EVOLUTIONARY CONSIDERATIONS

Asexual lineages may flourish briefly in some environments, but most appear to be dead ends with limited adaptive potential. From a phylogenetic perspective, obligately asexual plants and animals are little more than buds at the ends of branches that are fundamentally sexual. The rotifer class Bdelloidea is a notable exception. Although they appear to be strictly asexual, bdelloids have diversified into hundreds of morphologically distinct species that are classified into several families. We know of few other asexual taxa that have diversified in a similar way.

Bdelloids notwithstanding, numerous theories exist concerning the genetic, ecological, and evolutionary benefits of sex. Theories about the origin of recombination and meiosis in eukaryotic organisms are poorly understood and beyond the scope of this article. However, factors that favored the origin of sex (e.g., recombinational repair of DNA damage) need not be the same as those that currently maintain sex in higher organisms. Critical reviews of current hypotheses are provided in several of the listed references. Some major ideas related to the maintenance of sex in higher organisms are outlined in the following sections.

A. The Fisher-Muller Hypothesis (Sex Accelerates Evolution)

Adaptation by natural selection requires heritable genetic variation, and sexuality generates a new array of genotypes in each generation. Having more variation, sexual species should be able to adapt more quickly in a changing environment than asexual species. In the early 1930s, Ronald Fisher and Hermann Muller restated this hypothesis in genetic terms. Good mutations occur rarely (e.g., let the mutation rate, μ, be 10^{-8}). The probability of two good mutations arising simultaneously in the same asexual lineage is the vanishingly small product of these numbers (A^2 or 10^{-16}). It is more likely that two good mutations will come together in the same clone if

the first mutation spreads to near fixation before the second mutation arises in the same lineage. In a sexual population, however, the mutations can arise simultaneously in different individuals, and mixis will bring them together as each spreads to fixation.

Although the idea that sex is good for evolution seems intuitively satisfying, it suffers from several fundamental problems. It provides an advantage to sexual populations but not to the individuals that participate in sex. Sexual individuals will not spread at the expense of clones, unless the individuals also gain an advantage that compensates for the cost of males or meiosis. Furthermore, it is hard to see how sex could spread for the purpose of accelerating evolution of the species if evolution itself has no purpose. Evolution is a consequence of heritable variation among individuals and natural selection; it has no goals. Furthermore, evolving rapidly does not necessarily guarantee evolutionary success. Some "living fossils" such as *Limulus,* the horseshoe crab, and *Lingula,* an articulated brachiopod, have changed very little morphologically for hundreds of millions of years.

B. Muller's Ratchet (Sex Is a Way to Get Rid of Bad Mutations)

In 1960, Muller recognized another problem with the Fisher-Muller theory: The vast majority of expressed mutations are slightly deleterious. Recombination uncouples mutations and facilitates purging the bad ones. Muller suggested that slightly deleterious mutations will accumulate in asexual lineages and hitchhike along with the rare good mutations. Clones with the lowest genetic load may be lost due to genetic drift in finite populations. Except for the exceedingly rare back-mutation, the expected fate of an asexual population is to ratchet forward with deteriorating fitness. Other researchers have examined this problem in greater mathematical detail and refer to the mutational meltdown of clones. Despite the attractiveness of this argument, the evolutionary time scale for Muller's ratchet makes it difficult to imagine how it can compensate for the twofold cost of sex on an ecologically relevant time scale (but see Section IV,D).

C. The Tangled Bank (Sex Increases Niche Breadth)

Genotypic differences among individuals of a sexual species may contribute to more effective utilization of natural resources. In a heterogeneous environment, a sexual parent that produces diverse progeny may leave more offspring than a clonal parent that produces only one specialized type of offspring. Competition should be lower among the diverse sexual offspring than among clonal offspring. Thus, sexuals may gain a slight advantage over individual clones in a heterogeneous environment, but they may be eclipsed and replaced by an ecologically diverse assemblage of clones. Without considerable demographic stochasticity that leads to the random loss of clones, it is hard to see how this model can compensate for the twofold cost of sex.

D. The Red Queen (Sex Is Needed to Stay in Coevolutionary Race with Biological Enemies)

A consensus seems to be emerging that coevolutionary pressures from biological enemies (parasites, predators, and competitors) may provide sufficient ecological compensation for the costs of sex. Rapidly evolving microparasites (bacteria, viruses, etc.), because of their short generation times and vast numbers, will rapidly evolve means to avoid immune surveillance and exploit the most common host phenotypes. This provides rare host phenotypes a temporary advantage, until they rise in frequency and become the targets of newly evolved mechanisms of parasitic attack. Fitness of the host is frequency dependent, always favoring rare and different phenotypes, a cycle that maintains genetic polymorphism. Such a process would favor the parents of diverse offspring by spreading the risks of survival. This benefit is even more evident for species that brood their young and thereby increase the risk of contagion.

Red Queen processes may also facilitate the advance of Muller's ratchet. Frequency-dependent fitness will cause clones to cycle in abundance. Clones are more susceptible to random extinction when they are rare, and these losses may also remove clones

with the smallest load of deleterious mutations. Working together, the Red Queen and Muller's ratchet may result in a rapid decay of fitness that may account for the maintenance of sex on ecological time scales.

V. PARTHENOGENS AS STUDY ORGANISMS

Comparative studies of sexual and asexual organisms have provided considerable insight into the adaptive benefits of sex. Just as a physician studies deficiencies and diseases to understand the functioning of normal health, evolutionary biologists and ecologists study parthenogenetic clones as deviations from the normal sexual processes. Understanding the conditions under which asexuals prosper has provided insight into the short-term limitations of biparental sex. The overall biogeographical patterns of asexual organisms have likewise allowed biologists to reject some of models for the benefits of sex.

Efforts are also under way to compare the evolutionary longevity of closely related sexual and asexual taxa. Analyses of mitochondrial and nuclear genes provide a general picture that most asexual taxa, except bdelloid rotifers perhaps, arose recently and are relatively short-lived. Few asexual taxa have diversified to the extent that a taxonomist would be tempted to erect new species, genera, or families. For the most part, clonal diversity in asexual populations can be explained by recurrent origins of new clones from extant sexual progenitors. This observation leads to a surprising conclusion that the ecological success of many asexual taxa may depend on periodic recruitment of new genotypes from the sexual gene pool. Thus, sex, and periodic recombination, may also be essential for the ecological success and persistence of asexual populations.

See Also the Following Articles

ASEXUAL REPRODUCTION; CLONING; HYBRIDIZATION; MEIOSIS

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Parthenogenesis and activation of mammalian oocytes for *in vitro* embryo production: A review

Suresh Dinkar Kharche[1*], Hemant Shankar Birade[2]

[1]Central Institute for Research on Goats, Mathura, India
[2]Krantisinh Nana Patil College of Veterinary Science, Satara, India
Email: [*]kharche62@gmail.com

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ABSTRACT

Parthenogenesis is a form of asexual reproduction found in females, where growth and development of embryos occurs without fertilization by a male. Parthenogenesis occurs naturally in aphids, Daphnia, rotifers, nematodes and some other invertebrates but can also be induced efficiently in mammalian oocytes by providing appropriate stimuli *in-vitro*. Recently, parthenogenesis has attracted wide attention because of the role of activated oocytes in the field of research that have been described such as intra cytoplasmic sperm injection, cloning by nuclear transfer, somatic cell cloning, investigating culture conditions etc. & potential for deriving pluripotent stem cell lines and their differentiation into various cell lines that can be utilized for various tissue engineering applications. The parthenogenetically activated oocytes possess maternal genome and can developed in to either haploid, diploid or polyploidy embryos with the help of it we can analyze the possible role of all the genes involved in imprinting processes as well as the role the paternal genome plays during early embryo development by comparing them with fertilized embryos. Several methods are able to induce parthenogenetic activation through the elevation of cytoplasmic free calcium in oocytes. But one common, universal method or activation agents has not been developed for all species because the process is highly specific for each species. Therefore, activation step for each species need to be optimized accordingly. This review describes the general method of activation of mammalian oocytes and their genomic imprinting analysis.

Keywords: Epigenetic Modification; Genomic Imprinting; *In-Vitro* Maturation; Oocytes Activation; Parthenogenesis

[*]Corresponding author.

1. INTRODUCTION

Parthenogenesis is a phenomenon of undoubted biological interest which leads to the production of living young in many types of animals, as well as in plants. Parthenogenesis may initiate early embryonic development in mammals, and its lack of success in this class poses some fundamental and as yet unresolved problems regarding the significance of fertilization in the physiology of reproduction and embryonic development. This is one of the reasons why parthenogenesis is once again an area of active research. An individual resulting from the development of an unfertilized egg is variously referred to as "parthenogenone", "parthenogen", or "parthenote". The last term is American, while "parthenogenone" is preferred in the British literature [1]. Parthenogenesis is a reproductive strategy typical of lower species where a female gives birth to offspring's without a paternal contribution. On the contrary, parthenogenesis is not a form of natural reproduction in mammals even if mammalian oocytes, under appropriate stimuli, can undergo to parthenogenetic activation. Parthenotes can be efficiently obtained *in-vitro* with a variety of mechanical, chemical, and electrical stimuli using oocytes of several species at different stages along oocyte meiosis resulting in parthenotes with different chromosome complements [2].

Induced parthenogenesis; the experimental induction of parthenogenesis in mammals began with the pioneering studies of Pincus and his collaborators in the rabbit. Pincus and Enzman [3] showed that the extrusion of polar bodies could be induced *in-vitro* not only by contact with sperm suspension, but also by heat treatment or exposure to butyric acid and hypertonic solutions. Subsequently Pincus and Shapiro [4]; described the effect of cold treatment on unfertilized tubal eggs *in-vitro* and claimed not only an increased incidence of cleavage but also the production of a living young. There has since been abundant confirmation of the possibility of inducing parthenogenetic development in mammals by experimental procedures but none of the embryos so formed has survived beyond the embryonic period.

Using gene targeting, Kono *et al*. [5] were able to manipulate two imprinted loci H19/IGF2 and DLK1/ MEG3 to produce bi-maternal mice at high frequency and subsequently showed that fatherless mice have enhanced longevity and in April 2004, they used parthenogenesis successfully to create a fatherless mouse at Tokyo University of Agriculture Japan.

The general procedure of parthenogenetic embryo development is almost similar to the *in-vitro* embryo development of fertilized oocytes except the step of parthenogenetic activation with different activation agents which could either be electrical, chemical or other types and thus includes collection of ovaries, recovery of oocytes, *in-vitro* maturation of oocytes (IVM), activation of oocytes with different activation agents and finally *in-vitro* development of parthenogenetic embryos.

2. *In-Vitro* MATURATION OF MAMMALIAN OOCYTES

Although recent efforts in *in-vitro* embryo production system have led to advances in many steps, the efficiency of *in-vitro* embryo production is still low with regards to obtaining the fully matured oocytes, as only 30% of oocytes develop into blastocysts, probably because the *in-vitro* environment cannot mimic *in-vivo* environments and results in embryos with altered morphology and gene expression. Therefore, it is needed to standardize the culture conditions that mimic *in-vivo* embryo development [6]. The improvement of caprine culture system is highly desirable in terms of the production of preimplantation stage embryos for both biotechnological studies and embryo transfer technique [7]. The improvement of *in-vitro* culture systems are important for production of embryos with high developmental competence that are used in agricultural and biomedical research, and animal biotechnology [8].

Oocytes can either be retrieved from slaughter house ovaries within stipulated time or from the live animals by different techniques. Superovulation (hormonal treatment) of donor is routinely done to increase number of ova released by the ovary. Laparoscopic ovum pick up is one of the best techniques because of less adhesion problems compared to laparotomy or surgical oocytes collection from live animals. The cost of oocyte retrieval from live animal is high due to unpredictable results and low oocytes quantity. Therefore, slaughter house ovaries are attractive alternative as source for oocyte retrieval as they are less expensive and most abundant source of immature oocytes for large scale production of caprine embryos [6]. Lesser time taken between the slaughter and transport of ovaries is always preferred. Time interval between collection of ovaries and harvesting of oocytes also vary from 1 hour [9] to 3 - 4 h [10-12] without any detrimental effects at appropriate temperature. Most of the researchers observed a temperature range of 30˚C - 37˚C during transport of ovary to be the optimum for IVMFC of mammalian oocytes [6].

The obvious advantage of oocyte recovery technique is speed of operation, quality of oocytes, and quantity of oocytes. Therefore, different techniques of oocyte collection are employed in order to obtain maximum good quality oocytes. Follicle puncture, Ovary Slicing and follicle aspiration are routinely used techniques for recovery of oocytes from slaughter house ovaries. However, different views come from different researchers. Goat ovaries are relatively smaller in size therefore aspiration of follicular oocytes is difficult [13]. Therefore, in case of goat ovary, slicing and puncture techniques are most common in terms of obtaining good quality and quantity of oocytes [14]. The efficiency of *in-vitro* embryo production profoundly influenced by the number and quality of oocytes which successfully complete maturation. The ability to identify good quality oocytes prior to *in-vitro* maturation is the important consideration for *in-vitro* embryo production system. The existence of a healthy population of somatic cells surrounding the oocytes is mandatory to facilitate the transport of nutriaents and signals into and out of the oocytes [15].

Selection criteria of cumulus oocyte complexes (COCs) are extremely important for successful *in-vitro* maturation. Morphology of the cytoplasm and cumulus cell investment surrounding oocytes is the primary criteria for the grading and selection of oocytes for IVM. Normal oocytes should have cumulus cell investment surrounding the zona pellucid (ZP), absence of cracked zona pellucida and absence of vesicle in ooplasm [16].

Presence of more and compact layers of cumulus cells is considered better [17]. Retrieved oocytes could be graded as excellent (A), good (B), fair (C) and poor (D) quality depending upon the cumulus investment and cytoplasmic distribution. Excellent (A); Oocytes with more than 4 layers of bunch of compact cumulus cells mass with evenly granulated cytoplasm. Good (B): oocytes with at least 2 - 4 layers of compact cumulus cell mass with evenly granulated cytoplasm. Fair (C): oocytes with at least 1 layer of compact cumulus cell mass with evenly granulated cytoplasm. Poor (D): oocytes with no cumulus cells or incomplete layer of cumulus cell or expanded cells and having dark or unevenly granulated cytoplasm [12]. It is desirable to select A and B quality oocytes for IVMFC.

Granulosa Cell monolayer supports cytoplasmic maturation of growing oocytes, which is evident by a better maturation rate, active fertilization, improved cleavage rate and subsequently a higher rate of morula formation [12,18]. Culture media supplemented with gonadotropins (LH and FSH) and estradiol-17ß are reported to improve maturation rates significantly [12,19-21].

In-vitro maturation can be achieved by the supplementation of 20% estrous goat serum. Supplementation of 20% EGS or 10% FBS + 3 mg/ml BSA in TCM-199 medium could also be used to achieve maturation followed by fertilization, embryo culture, and subsequent embryo transfer resulted in successful birth of caprine kid [10,16,22]. In our previous study we found slightly better *in-vitro* maturation rate in the presence of antioxidant ß-mercaptoethanol as compared to base medium (TCM-199 with NCS and 3 mg/ml BSA) but lesser than base medium containing hormones (LH-5µg/ml, FSH-5 µg/ml, Estradiol 1 µg/ml) [21]. A significant positive effect of epidermal growth factor (EGF) on IVM of oocytes was reported in cattle [23], sheep [24], pigs [25], buffalo [26] and in goats [21].

Further the time required for *in-vitro* maturation of various mammalian oocytes varies depending upon their own complexity. The time required for *in-vitro* maturation of mouse oocytes is 18 h [27]; Goat, 27 h [6], Rabbit, 14 - 15 h [28], Cat, 36 h [29], Camel, 24 - 48 h [30]. In that regard, bitches ovulate immature oocytes, and the oocytes require 2 - 5 days for meiotic maturation within the oviduct and remain surrounded by cumulus cell mass, unlike other mammals [31]. Good quality Buffalo oocytes were matured for 22 - 24 hrs. [32,33] whereas Procine oocytes, 22 - 24 hrs [34].

In compare to the majority of species, porcine oocyte maturation occurs over a longer period and this has resulted in the development of a two-stage maturation process. In the first step COCs are cultured in medium supplemented with hormone(s) such as eCG and hCG or HMG for 20 - 22 h in order to enhance nuclear maturation, in the second step, COCs are cultured in maturation medium without hormonal supplement(s) for 18 - 24 h. The removal of hormones is thought to slow nuclear maturation and enhance cytoplasmic maturation. North Carolina State University medium 23 (NCSU 23) supplemented with 10% porcine follicular fluid (pFF); NCSU 37 medium + 10% pFF; TCM 199 medium + 10% pFF and TCM 199 supplemented with 0.1% PVA are frequently used as a basic maturation medium for *in-vitro* maturation of procine oocytes [32]. However, Sow oocytes were significantly better than gilt oocytes when maturation rate, development to blastocyst and mean blastocyst cell number were compared.

Maturation can be judged directly by staining their nuclear and chromatin structure and/or by the ability of the oocytes to be fertilized or activated [6]. Cytoplasm of the oocyte may play a crucial role in assembling the correct metabolic environment for production of sufficient energy for cellular functions during maturation, cleavage and blastocyst formation [18]. However ooplasmic changes occur during oocytes maturation are still difficult to evaluate. Cytoplasmic maturation refers to the other maturation events that prepare the oocytes for fertilization and preimplantation development [35]. Degree of cumulus cells expansion can be used as a morphological indicator for maturation of oocytes. So, it can be said that expanded cumulus cells indicates mature and good quality oocytes while a compact cumulus cells characterizes immature oocytes [36]. When fully grown oocytes are released from their follicles to the culture medium they resume meiosis spontaneously in maturation medium. The reduced development of *in-vitro* derived zygote suggest that the conditions of IVM do not support cytoplasmic maturation, so it is very important that the improvement of the *in-vitro* maturation systems for oocytes aimed at defining *in-vitro* conditions that are more similar to the *in-vivo* environment [37]. However, development is still compromised compared to oocytes matured *in-vivo* and further research is required to optimize maturation in all species. Therefore development of appropriate IVM culture conditions that can mimic *in-vivo* culture condition for each type of mammalian oocytes is essential.

3. PARTHENOGENESIS

Meiotic maturation of oocytes starts during the fetal development of mammalian females, but is arrested at the late diplotene stage. The ability to resume meiosis and to continue in maturation beyond this stage is acquired gradually during the subsequent period of oocyte growth. In fully grown oocytes, meiosis continues after germinal vesicle breakdown (GVBD) through the stages of metaphase I, anaphase I and telophase I to the stage of metaphase II, when meiosis is again arrested. The ability to pass through all these stages is designated as full meiotic competence. During the growth period, oocytes pass through a stage in which meiotic competence is only partially developed [38]. These oocytes acquire the ability to undergo germinal vesicle break down (GVBD) and to enter metaphase I stage. However, they are unable to exit from metaphase I stage, to reach metaphase II stage, and to complete meiotic maturation [39-41]. Parthenogenetic activation of matured oocytes is a valid tool to assess their cytoplasmic maturation and quality. Furthermore, identification of an optimal protocol for oocyte activation is required for the production of genetically identical animals by somatic cell nuclear transfer.

Present methods for parthenogenetic embryo production *in-vitro* depend on the use of oocytes with full meiotic competence, which are present in the ovary in limited numbers. Numerous populations of follicles with growing oocytes that have partially developed meiotic competence cannot be used for these purposes. However, embryos produced from these oocytes could be used for breeding, production of cloned or transgenic animals, or for preservation of endangered breeds. To this end, cul-

ture systems for *in-vitro* growth and acquisition of full meiotic competence of mammalian oocytes have been developed [42,43]. However, the processes involved in the acquisition of full meiotic competence are not fully understood. Sedmíková *et al*. [44] demonstrated that drugs elevating intracellular calcium levels can overcome the meiotic block in oocytes with partially developed meiotic competence and can induce their maturation to the metaphase II stage. To achieve these results they used cyclopiazonic acid, the inhibitor of calcium-dependent ATPases, which elevates intracellular levels of free calcium ions through the mobilization of intracellular calcium deposits [45,46]. Successful activation has been achieved by a range of treatments including electrical stimulation, as well as chemicals such as strontium in mouse, ionomycin, calcium ionophore in cattle and sheep. In addition, there are many factors influencing efficient activation; concentration of chemical agents, duration between fusion and activation, activation media, strength of electric stimulation, post-treatments such as cytochalasin B or D (CB, CD), cycloheximide (CHX), or dimethylaminopurine (DMAP) etc. Several methods are able to induce parthenogenetic activation through the elevation of cytoplasmic free calcium in oocytes. One common, universal method or activation agents has not been developed for all species because the process is highly specific for each species therefore, combination of activation agents is also applied. Similarly concentration as well as incubation time of the activation agents are also species specific and need to be optimized.

Parthenogenesis in bovine oocytes can be induced with an electrical pulse [47,48], ethanol [49-51], calcium ionophore A23187 [52,53], cycloheximide (CHX) [54,55], 1,4,5-inositol triphosphate [56], ionomycin [57,58], or strontium [59]. When ethanol was used in combination with CHX, the success rate for parthenogenesis was further enhanced [51,54]. Similarly, better results were obtained after oocytes were first treated with ethanol or ionomycin prior to treatment with DMAP [60]. 9% ethanol (5 min) followed by 6-DMAP (4 h) promoted optimal parthenogenetic activation of bovine oocytes [61].

Activation response in bovine oocytes by several activation agents has been demonstrated to be oocyte age dependent also. Namely, the development to the pronuclear stage was investigated following activation treatment by ethanol [49,62] calcium ionophore [52,63] electric pulse [52,62,64,65] or cycloheximide [62-64]. While less than 40% of the young or aged (23 - 42 h) *in-vitro* matured oocytes were activated [49,64,66]. The relation between activation and aging of recipient oocytes is a factor which affects the development of activated embryos.

Aging of oocyte decreases the fertilization rate and the subsequent development [67,68]. Therefore, using young recipient oocytes is attractive and may increase the overall efficiency of cloning procedures if the oocytes can be activated adequately. Aoyagi *et al*. [69] demonstrated that, reconstituted embryos had a high developmental rate to the blastocyst stage when a combination treatment of Ca-ionophore, electric pulse and cycloheximide was used to activate young oocytes. Combined ethanol and cycloheximide treatment has been reported to effectively (over 90%) activate freshly matured bovine oocytes [51,54,62,70]. Therefore, the combined activation of young oocytes leads to a more efficient development of bovine embryos.

Among several activating artificial agents, some promote intracellular calcium increase, e.g. strontium [71, 72], ionomycin [73], electric pulse [74], and ethanol [73], and others inhibit protein synthesis e.g. cycloheximide [75] or protein phosphorylation e.g. 6-DMAP [73]. In the case of intracellular calcium releasing treatments, ionomycin induces a single increase and is frequently used in combination with protein phosphorylation inhibitors [73]. The great disadvantage of using the protein kinase inhibitors or the protein synthesis inhibitors is that these inhibitors do not specifically inhibit the activity of a particular kinase or the synthesis of a specific protein that control cell-cycle progression. However, they inhibit the activity of several kinases or the synthesis of several proteins that may be involved in other cell functions, whose inhibition may have a deleterious effect on the subsequent cellular events after oocyte activation [76]. Moreover, the calcium oscillations triggered by the sperm cells function not only in inducing resumption of meiosis but also in many other events [77]; for example, recruitment of specific maternal RNAs [78,79], which is essential for activation of zygotic genome [80] and may be extended to other unknown functions. Therefore, a new activation regimen without using either protein synthesis or protein phosphorylation inhibitors but with two trigger agents for single calcium increase effectively improved blastocyst yield.

Strontium (divalent cation), has also shown to promote multiple free calcium oscillations (similar to fertilization) in mouse oocytes [81]. Therefore it can also be used to activate oocytes of various animals including cattle. Meo *et al*. [82] successfully applied strontium efficiently for bovine oocyte activation at 20 mM in Ca^{2+}- and Mg^{2+}-free TALP medium for 6 h. Hosseini *et al*. [83]; studied fusion pulses along with a chemical activation protocol and sequential use of Ca^{2+} immobilizing agents, which may benefit the activation outcome in bovine oocytes. A combination of single, double or triple compounds of EP (electrical pulse), sequential combinations of calcium ionophore (CI), ethanol (ET), strontium (SR) along with 6-DMAP on *in-vitro* matured bovine oocytes showed the best cleavage rates with double (SR-CI, 84.4%), triple

(CI-SR-ET, 79.4%) and single (CI, 73.7%) compounds, respectively, which were not significantly different with each other and with *in-vitro* fertilized (85.5%) oocytes. The highest blastocyst rates were gained with ET-SR (24.5%), SR-CI-ET (20.4%) and CI (24.5%) accordingly which were not significantly different with each other but significantly lower than IVF (47%). Embryo cell counting further confirmed reasonably better quality of blastocysts produced using double, triple and single compounds.

In buffalo, ethanol has been employed as an agent [84], which activates oocytes by promoting the formation of inositol 1,4,5-triphosphate (IP3) at the plasma membrane and the influx of extracellular Ca^{2+} [85], causing a large, single rise in intracellular Ca^{2+} concentration [72]. Inomycin is another popular activating agent currently used in buffalo nuclear transfer protocols [86], which induces repetitive transient rises of Ca^{2+} lasting for several hours, probably by displacing bound Ca^{2+} in the oocytes. Electrical stimulation has also been used for activation of *in-vitro* matured oocytes in buffalo [87,88], whereas ethanol, ionomycin and calcium ionophore were used as chemical activators in buffalo [89,90]. Within the ethanol and ionomycin activation groups, ethanol supported the highest development in terms of cleavage (71:4 ± 7:8 versus 59:4 ± 10:7) and morulae-blastocysts rate (32:6 ± 6:5 versus 25:7 ± 8:3). Similarly, ethanol activation gave better results than the IVF control group, with higher cleavage rate (71:4 ± 7:8 versus 55:8 ± 5:8, respectively) and a higher proportion of oocytes developing into morulae-blastocysts (32:6 ± 6:5 versus 22:9 ± 7:5, respectively). It was also observed that aging negatively affects post-parthenogenetic and post-fertilization development [89]. Interestingly, despite the similar maturation rate of buffalo (87%) and cattle (94%) oocytes, the cleavage rate in buffalo oocytes is poor (64% versus 84%) or very poor (9% - 45%) and may be attributed to poor activation by sperm at the time of fertilization [91,92]. To ascertain possible reasons for low cleavage following IVF and to identify the role of sperm in the process of fertilization and cleavage, Mishra *et al*. [33] compared chemical activation protocols on *in-vitro* matured oocytes with IVF (natural activation) and observed that cleavage rate was significantly higher following ET + DMAP, ET + CHX and ET + CHX + DMAP activation (52.5%, 52.5% and 44.4%, respectively) compared to IVF (36.5%, 23.4% and 26.8%, respectively). Blastocyst development (30.9% versus 15.2%) was also significantly higher following ET + CHX + DMAP activation than IVF. Thus, buffalo oocytes had better inherent developmental competence and that the poor cleavage and embryo development following IVF may be due partly to the poor quality of frozen/thawed sperm, improper sperm capacitation and/or fertilization.

The literature on activation protocols for goat oocytes is limited therefore; effective activation protocols need to be developed. Ongeri *et al*. [93] compared the development of IVF goat embryos with those of non-fertilized parthogenetically developing oocytes activated by treatment with either ionomycin or ethanol, both followed by immediate exposure to 6-diethylaminopurine (6-DMAP). In both shipped and non shipped oocytes, parthenotes developing from ionomycin and ethanol activated oocytes had significantly greater blastocyst development compared to IVF embryos. Guo *et al.* [94] evaluated the ionomycin, strontium and electrical pulse for the effecttive activation and parthenogenetic development of goat oocytes. The activation of oocytes by ionomycin combined with 6-dimethylaminopurine, strontium plus cytochalasin B and electrical pulses combined with cytochalasin B revealed 79.3% - 81.6%, 2.2% - 78.8% and 65.5% of the oocytes cleaved and 16.2% - 24.8%, 0% - 15.6% and 11.1% of the cleaved embryos developed into blastocysts, respectively. In our lab, we tried to optimize the protocols for caprine oocytes activation through comparing the effectiveness of different concentration of ethanol treatment on the activation and subsequent development of oocytes. In the Experiment, matured oocytes were treated with single activation agent *i.e.* Ethanol with concentration ranging from 1%, 3%, 5%, 7% & 9% for 5 min. The cleavage rates were gradually higher with higher concentration of ethanol treatment. Development of the embryo up to morula stage were also follow the same trend upto 7% ethanol treatment and decreased at higher concentration *i.e.* at 9% ethanol. Blastomeres were also shows less compaction in all other treatments including 9% ethanol concentration. These results suggested that ethanol treatment (7% for 5 min) is most favorable for parthenogenesis of caprine oocytes and its further development *in-vitro* [95]. The use of Ca ionophore (5 μM) and 6-DMAP (2 mM), activation of caprine oocytes for the production of zona and zona-free parthenogenetic embryos in three different culture media revealed that zona parthenogenetic hatched blastocysts were highest in RVCL (6.8% ± 0.9%) as compare to mSOF (1.2% ± 0.7%) and EDM (5.5% ± 0.7%) ($P < 0.05$) media, respectively. Similarly, zona-free parthenogenetic blastocyst, formation was greater in the RVCL (8.8% ± 0.9%) as compare to mSOF (5.6% ± 0.5%) and EDM (5.1% ± 0.8%) ($P < 0.05$) media, respectively [96].

Several protocols have been used to successfully create parthenogenetic sheep embryos [73,97-99]. Grazul-Bilska *et al*. [100] validated and optimized the methodologies necessary to create parthenogenetic sheep embryos for future studies of placental development in normal and compromised pregnancies. The oocytes were activated using ionomycin (a calcium ionophore) and 6-dimethylaminopurine (DMAP; a protein kinase inhibi-

tor). Activation of oocytes in serum-free medium resulted in minimal cleavage rates. However, replacement of ionomycin with ethanol treatment resulted in decreased blastocyst formation (from 58% to 19%, respecttively) but not cleavage rates (83% and 81%, respectively [73]. It is known that ionomycin induces smaller and thus less cytotoxic rise of intracellular calcium-whereas ethanol induces extracellular as well as intracellular release of calcium. In another study, the rate of blastocyst formation was 25% after oocyte stimulation with direct current pulses and treatment with cycloheximide plus cytochalasin B [101]. Thus, the activation protocol may have a profound effect on success of the oocyte activation to obtain parthenogenetic embryos. Shirazi *et al.* [102] compare the effect of time of parthenogenetic activation (22 hr versus 27 hr after *In Vitro* Maturation-IVM) on in vitro development of ovine oocytes using either single (Ionomycin 5 μM for 5 min or Ethanol 7% for 7 min) or combined (ionomycin and ethanol with 6-DMAP 2 mM for 3 hr) activation treatments. The cleavage and blastocyst rates in single-treated groups were positively influenced by the extension of duration of IVM (27 hr). A trend of decreased numbers of total cells and ICM was observed in slightly aged oocytes. Moreover, developmental potential of ovine parthenotes, especially in young oocytes, was improved by the addition of 6-DMAP to the activation regimen.

In addition to the above mentioned oocyte activation factors, several other factors can activate oocytes to induce parthenogenetic development, including chilling or warming, exposure to colchicine, exposure to electric pulses in the presence of Gluta MAX-I, pricking, certain anesthetics, and factors disturbing the balance between free calcium and the state of the cycloskeletal system [103,104].

4. GENOMIC IMPRINTING ANALYSIS OF PARTHENOGENETICALLY ACTIVATED EMBRYOS

When the mammalian oocyte is fertilized with sperm, it receives the paternal genetic materials. The paternal alleles, like the oocyte alleles, have been subjected to epigenetic modifications during gametogenesis that cause a subset of mammalian genes to be expressed from one of the two parental chromosomes in the embryo. This regulatory mechanism is termed genomic imprinting [105, 106]. Additional epigenetic processes also occur during early development after fertilization [73]. Thus, the maternal and paternal genomes are not functionally equivalent, which is why both a maternal and a paternal genome are required for normal mammalian development. Mammalian parthenotes are able to undergo several cycles of cell division after oocyte activation, but never proceed to term, arresting at different stages of development, depending on the species [50,73,105,106].

Success rates and viability of parthenogenetic embryos appear to be organism dependent. Mouse parthenotes are capable of developing beyond the postimplantation stage *in-vivo* [107,108]; porcine parthenotes have developed up to post-activation day 29 (limb bud stage, past the early heart beating stage); rabbit parthenotes until day 10 - 11 [109] and primates (Callithrix jacchus) have only been shown to implant stage [110]. The reason for this arrested development is believed to be due to genetic imprinting. Since all genetic material in parthenotes is of maternal origin, there is no paternal imprinting component and this prevents proper development of extraembryonic tissues whose expression is regulated by the male genome [111].

Uniparental embryos, such as parthenotes or androgenotes, have been used to study imprinting processes as well as the role the paternal genome plays during early embryo development [112]. Since diploid parthenotes (DPs) and fertilized embryos show similar development, at least to the blastocyst stage, their gene transcription patterns during early developmental processes may not differ markedly. However, there may be some more subtle differences in that fertilized embryos may express Y-chromosome-linked genes and imprinting genes during early development, unlike the DPs. Comparison of the gene expression patterns of the fertilized embryo and the DP parthenote may thus illuminate the role(s) paternal genes play in later embryonic development. Compared to DPs, fewer haploid parthenotes (HPs) cultured *in-vitro* reach the blastocyst stage and those that does have lower cell numbers [28,113]. The reasons for this limited developmental potential of mammalian HPs are not clear. One possibility is that the lack of genetic component(s) in HPs may increase the duration of the cell cycle and consequently slow their development [28]. This explanation is supported by the observation that mouse HPs develop *in-vitro* more slowly than DPs during the preimplantation period [114]. Another possible explanation is that the low DNA content in HPs may not be sufficient to control the gene expression network, which could result in apoptosis [28,115,116] or the failure of developmental processes during preimplantation development.

To gain insights into the roles the paternal genome and chromosome number play in pre-implantation development, cultured fertilized embryos and diploid and haploid parthenotes (DPs and HPs, respectively), and compared their development and gene expression patterns. The DPs and fertilized embryos did not differ in developmental ability but HPs development was slower and characterized by impaired compaction and blastocoel formation. These results are consistent with previous reports that indicated HPs are developmentally retarded

and show slow development in mice [28,114]. While it remains unclear why HPs show more limited and slower development, it may be speculated that at least part of the reason may involve the difficulties HPs have in compacting. Compaction during embryonic development involves the formation of tight junctions between outer cells, which permits selective ion transport and facilitates blastocoel formation [117]. Thus, the incomplete compaction of HPs may be responsible, at least in part, for their impaired development to the blastocyst stage. Microarray analysis revealed that fertilized blastocysts expressed several genes at higher levels than DP blastocysts; these included the Y-chromosome-specific gene eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked (Eif2s3y) and the imprinting gene U2 small nuclear ribo nucleoprotein auxiliary factor 1, related sequence 1 (U2af1-rs1). It is found that when DPs and HPs were both harvested at 44 and 58 h of culture, they differed in the expression of 38 and 665 genes, respectively. When differentially expressed genes in the HPs as compared to the DPs at 58 h after activation were analyzed with regard to their putative molecular function. 176 highly expressed and 158 lower expressed genes were unclassified. Of the remainder, it was found that 12 highly expressed and 9 lower expressed genes were related to cell adhesion/cell junction/cytoskeletal-functions.

Moreover, compared to the 58 h DPs, the 58 h HPs showed lower expression of more nucleic acid-binding proteins, oxidoreductase, transcription factors, selected regulatory molecules, and transferase, and highly expression of more receptors, transcription factors, nucleic acid-binding proteins, kinases, and selected regulatory molecules.

However, when DPs and HPs were harvested at the midpoints of 4-cell stage (44 and 49 h, respectively), no differences in expression was observed. Similarly, when the DPs and HPs were harvested when they became blastocysts (102 and 138 h, respectively), only 15 genes showed disparate expression. These results suggest that while transcripts needed for early development are delayed in HPs, it does progress sufficiently for the generation of the various developmental stages despite the lack of genetic components.

Genomic imprinting, a specific genetic mechanism in mammals, plays important roles in the regulation of fetal growth, development, placental function, and postnatal behavior [118-120]. It endows some genes with different "imprints", which lead to their differential expression in fetuses and/or placenta and regulate the transfer of nutrients to fetus and placenta from the mother [121].

The establishment of genomic imprinting is controlled by DNA methylation, histone modifications, noncoding RNA, and specialized chromatin structure; DNA methylation is thought to be a major factor [122-124]. Specific DNA methylation in the differentially methylated regions (DMRs) of parental origin allows the discrimination between the maternal and the paternal alleles and leads to monoallelic expression of imprinted genes [125]. Uniparental fetuses, including parthenotes and androgenotes, show disrupted expression of several imprinted genes, such as Snrpn, Peg3, H19, and Gtl2 [126,127]. Studies in mouse uniparental embryos have revealed that the paternal genome is more important for the development of the extraembryonic tissues, while the maternal genome is more essential for fetal development. These distinctive differences are the result of genomic imprinting [128]. Parthenogenetic fetuses die by day 10 of gestation [129]. Likely, the cloned animal fetuses exhibit a high rate of developmental abnormalities due to inefficient epigenetic reprogramming of the donor nucleus within enucleated oocytes [130-132]. The aberrant epigenetic modifications caused by inefficient reprogramming everely undermine the developmental potency of cloned embryos [132-134]. But to date, our knowledge about the molecular mechanism of epigenetic reprogramming is still very limited [135]. Compared to the laborious manipulation of somatic cell nuclear transfer, the mouse parthenogenetic embryo is a most suitable alternative to study the events of methylation imprints. Similar to mouse parthenogenetic embryos, the aborted cloned bovine fetus also exhibits disrupted expression of imprinted genes and aberrant methylation imprints [136, 137]. To obtain further insight into the dynamics of methylation imprints during development of diploid parthenogenetic mouse embryos, [138] determined the methylation status of DMRs of three maternally imprinted genes and two paternally imprinted genes using bisulfite mutagenesis sequencing methods. They showed that the maternally imprinted genes Snrpn and Peg1/Mest were nearly unmethylated or heavily methylated, respectively, in their differentially methylated regions (DMRs) at the two-cell stage in parthenogenetic embryos. However, both genes were gradually de novo methylated, with almost complete methylation of all CpG sites by the morula stage in parthenogenetic embryos. Unexpectedly, another maternally imprinted gene, Peg3, showed distinct dynamics of methylation during preimplantation development of diploid parthenogenetic embryos. Peg3 showed seemingly normal methylation patterns at the two-cell and morula stages, but was also strongly de novo methylated in parthenogenetic blastocysts. In contrast, the paternally imprinted genes H19 and Rasgrf1 showed complete unmethylation of their DMRs at the morula stage in parthenogenetic embryos. These results indicate that diploid parthenogenetic embryos adopt a maternal-type methylation pattern on both sets of maternal chromosomes and that the aberrantly homogeneous

status of methylation imprints may partially account for developmental failure.

5. CONCLUSION

A variety of activation stimuli and activation protocols have resulted in the production of viable embryos for parthenogenetic as well as somatic cell cloning research in a range of species. However, both the stimuli and the protocol used must be optimized for use in each species and there is a need for understanding the mechanism and effects by various activation methods. Since the birth of Fatherless mouse (Kaguya) the first viable parthenogenetic mammal in 2004 in Japan, significant advances have been made in the field of parthenogenetic research in order to understand the molecular processes involved during genomic imprinting process which is the main (perhaps the only) barrier to parthenogenetic development in mammals, in which the individual contains no paternal genetic material. Development of Parthenogenetic embryos is a multifactorial process and advances in all areas will contribute to simplifying and improving the efficiency of the technique.

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THE SCIENCES

Korean Cloned Human Cells Were Product of "Virgin Birth"

Fraudulent cloned cells were likely the first example of a human egg turned directly into stem cells

By JR Minkel on August 2, 2007

Researchers say they have confirmed suspicions that embryonic stem cells claimed to be extracted from the first cloned human embryo by discredited South Korean scientist Woo Suk Hwang actually owe their existence to parthenogenesis, a process in which egg cells give rise to embryos without being fertilized by sperm.
A series of genetic markers sprinkled throughout the cells' chromosomes show the same pattern found in parthenogenetic mice as opposed to cloned mice, according to a report published online today in the journal *Cell Stem Cell*.

The result suggests that, although Hwang deceived the world about achieving the first

The result follows on the heels of an announcement last month by another California stem cell company, International Stem Cell Corporation (ISC) in Oceanside, that it had successfully achieved human parthenogenesis for the first time. Last year, Italian researchers claimed to have achieved the same feat but have yet to publish their results.

"The fact that this has now been achieved by two independent groups gives me a far greater degree of confidence," Lanza says.

The new finding brings a measure of closure to a story that first rocked the science

falsely reported creating 11 cell lines genetically matched to their donors.

Unsolved Mysteries

A cloned cell should be identical to its donor, but the probe found that of 48 common genetic variations, or markers, present in the 2004 cells, eight did not match their apparent donor. Investigators raised parthenogenesis as the most likely explanation but could not be certain.

Later, during a chance discussion with European colleagues, stem cell researcher

The DNA of any two people will differ on average at one of every 1,000 subunits, or base pairs, Daley says. When a chromosome from a sperm cell joins with that of an egg, these single nucleotide polymorphisms (SNPs or "snips") tend not to match each other.

The same goes for cloned cells. But in contrast, pairs of matching chromosomes in parthenogenetic cells tend to match one another in the middle and differ near the ends because of a genetic mixing process called recombination. In their paper, Daley and colleagues report that the SNPs in the Korean cell line do indeed match toward the center of the chromosomes, similar to five parthenogenetic mouse cell lines that the team created for comparison.

Jeffrey Janus, president and director of research for ISC, agrees that "Dr. Hwang's cells have characteristics found in parthenogenetic cells" but remains cautious, saying "it needs more study."

The Irony of It All

Stem cell experts say that Hwang and his team probably had no clue what they had achieved, because if they had they would have claimed credit for it.

"I think this ... is every bit as exciting as the SCNT they were claiming," says stem cell researcher Kent Vrana of Pennsylvania State University, who pioneered parthenogenesis in monkeys. "Parthenotes by their very nature are nonviable embryos, so you're not destroying embryos, which has some ethical advantages."

removal of the DNA," Daley says, "but obviously they didn't."

The injection of the donor nucleus could have failed if the injecting needle pulled it back out when withdrawn from the egg or if the egg somehow rejected the introduced nucleus, Vrana says.

Hwang's group purported to rule out parthenogenesis as an explanation in part by showing that two genes normally activated by paternal DNA were inactive in the cells. But Daley says such experiments are easy to misinterpret and are less conclusive than sequencing SNPs.

"I think they were just so blinded by what they hoped to accomplish, they missed it," Vrana says.

and surprisingly successful: out of some 50 donated eggs, the company grew six cell lines. Parthenogenesis in monkeys typically works only once every 90 eggs, he says.

Banking on Parthenotes

The therapeutic potential of parthenogenetic cells remains to be seen. The lack of imprinting from the paternal DNA may cause the cells to behave abnormally as they develop. Furthermore, they must have matching immune proteins to be transplanted back into a donor.

In principle, tissue banks of parthenogenetic cell lines could include enough different immune protein combinations to treat up to half of the U.S. population—men as well as women—Lanza says. But he adds that if human parthenotes routinely contain as many genetic mismatches as the Korean cells, the number of eggs needed to create such a bank could be prohibitively large.

Daley says his group hopes to acquire donated eggs from women with inherited diseases and use parthenogenesis to create cell lines to study those disorders. In the future, researchers will have to determine whether similar cells are safe and effective when transplanted.

"We're a long, long way," Daley says, "from realizing therapeutic uses of these cells."

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Parthenogenesis in a large-bodied requiem shark, the blacktip *Carcharhinus limbatus*

D. D. Chapman*†, B. Firchau‡ and M. S. Shivji§

*Pew Institute for Ocean Science, Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Cswy, Miami, FL 33149, U.S.A., ‡Virginia Aquarium & Marine Science Center, 717 General Booth Boulevard, Virginia Beach, VA 23451, U.S.A. and §Guy Harvey Research Institute, Oceanographic Center, Nova Southeastern University, 8000 North Ocean Drive, Dania Beach, FL 33004, U.S.A.

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Genetic evidence is provided for parthenogenesis in a large-bodied shark, the blacktip *Carcharhinus limbatus*, from the speciose and commercially important family Carcharhinidae, the first verified case of asexual development in this lineage and only the second for any chondrichthyan. The parthenogenetic embryo exhibited elevated homozygosity relative to its mother, indicating that automictic parthenogenesis is the most likely mechanism. Although this finding shows that parthenogenesis is more common and widespread in sharks than previously realized and supports the early existence of parthenogenetic abilities in vertebrates, the adaptive significance of automixis in these ancient fishes remains unclear.

Key words: automixis; Carcharhinidae; diversity; evolution; genetic management.

Automictic parthenogenesis (automixis) is a type of asexual reproduction characterized by fusion of an ovum and its sister polar body, producing a diploid zygote with elevated homozygosity compared to its mother (Schuett *et al.*, 1998). Experimental evidence for vertebrate automixis was recently obtained for a bony fish (Lampert *et al.*, 2007), and it is considered very likely to be the mechanism often underlying facultative parthenogenesis in more derived vertebrate lineages [reptiles and birds (Olsen, 1975; Schuett *et al.*, 1998; Watts *et al.*, 2006; Lampert *et al.*, 2007)]. Automixis is also postulated as the mechanism behind the first confirmed case of parthenogenesis in the most ancient jawed vertebrate lineage, the Chondrichthyes (sharks, batoids and chimeras), where a parthenogenetic embryo with elevated homozygosity was recently described in a small-bodied hammerhead shark *Sphyrna tiburo* (L.) (Sphyrnidae) (Chapman *et al.*, 2007). Since automixis is easily overlooked in wild vertebrate populations and there is only a rudimentary understanding of its breadth of evolutionary occurrence and frequency (Chapman *et al.*, 2007;

†Author to whom correspondence should be addressed. Tel.: +1 305 421 4908; fax: +1 305 421 4077; email: dchapman@rsmas.miami.edu

Lampert *et al.*, 2007), it is currently of general biological interest to determine how widespread and common it is among sharks.

There are a growing number of instances where captive female sharks have produced apparently normally developed offspring despite extended periods of isolation from conspecific males (Castro *et al.*, 1988; Voss *et al.*, 2001; Heist, 2004), suggesting that asexual development could be more common and evolutionarily widespread in this lineage than reflected by the single genetically verified case in *S. tiburo* (Chapman *et al.*, 2007). Almost all these suspect cases involve oviparous (egg-laying) species that have small adult body sizes (<1·2 m total length, L_T). Although this may reflect a bias towards the practicality of keeping smaller shark species in captivity, it could also indicate that small-bodied sharks have evolved parthenogenesis as a means to avoid reproductive failure in situations when males are scarce within isolated habitat patches since small shark species tend to have more limited dispersal capabilities than larger species (Musick *et al.*, 2004). An intriguing possible case of parthenogenesis in a large-bodied, highly migratory shark was revealed on 30 May 2007 during the necropsy of *c.* 9 year-old captive female blacktip shark *Carcharhinus limbatus* (Müller & Henle) that had failed to fully revive after being tranquilized during a routine veterinary examination. The necropsy revealed a single, well-developed female embryo, even though the adult female had been isolated from conspecifics for all 8 years of its captivity. Exhibiting placental viviparity, female *C. limbatus* typically reach sexual maturity around age 7 years (Killam & Parsons, 1989) and give birth to multiple offspring every other year after a gestation period of 12 months (Castro, 1996). This indicates that the embryo was probably produced during the first or second ovulation of this female. The adult female had shared the display tank with only one other carcharhiniform shark, an adult male sandbar shark *Carcharhinus plumbeus* (Nardo) that was also captured locally. Despite daily observations by aquarium curators and routine veterinary examinations of the female, there was never any physical evidence of copulation between these two sharks (*i.e.* mating wounds or observations of mating).

Given the captivity circumstances and the case of automixis in the carcharhiniform *S. tiburo* (Chapman *et al.*, 2007), the hypothesis that the *C. limbatus* embryo had resulted from automictic parthenogenesis was tested (*i.e.* with the expectation that it would have no paternal *C. limbatus* or *C. plumbeus* alleles and would exhibit elevated homozygosity rather than being an exact genotypic match to its mother). Tissue samples (fin clips) were obtained from the *C. limbatus* mother and her embryo and stored in 95% ethanol. Following genomic DNA isolation (DNeasy kit; Qiagen Inc., Valencia, CA, U.S.A.), five microsatellites (three to 20 alleles per locus) previously isolated from the genome of *C. limbatus* were amplified in both individuals (locus-specific protocols and diversity are given by: Keeney & Heist, 2003). The polymerase chain reaction (PCR) products were resolved on an AB 3130 DNA analyser and scored in the programme GENEMAPPER 3.7 (Applied-Biosystems Inc., Foster City, CA, U.S.A.). All reactions were replicated and the genotypes scored by two experienced DNA analysts.

The embryo's composite five-locus genotype contained no paternal alleles and every locus exhibited homozygosity for a maternal allele (Table I), both findings

concordant with automixis. The probability of obtaining an embryo that is homozygous for all five loci assuming sexual reproduction within the source *C. limbatus* population (U.S. Atlantic Ocean) was estimated by multiplying the frequencies of observed homozygosity from population genetic data given in Keeney *et al.* (2005). This probability is extremely low ($P < 0{\cdot}0001$), permitting rejection of an alternate hypothesis of sexual reproduction between a male *C. limbatus* and the mother while she was a small juvenile prior to capture followed by an extraordinarily long period of sperm storage. The alternate hypothesis that the embryo was sired by the male *C. plumbeus* can also be rejected because four of the five microsatellites amplify both of these carcharhinid species (Keeney & Heist, 2003) with the expectation that paternal *C. plumbeus* alleles should be observable in the embryo's composite genotype. Although it would have been desirable to have also genotyped the *C. plumbeus* to confirm that it amplifies at these loci, the animal is too large to remove safely from the tank for DNA sampling. It originated, however, from the same *C. plumbeus* population where these loci were shown to cross amplify (Keeney & Heist, 2003). A species-specific *C. plumbeus* PCR-primer (Pank *et al.*, 2001) failed to amplify genomic DNA from the embryo, while a *C. limbatus*-specific primer succeeded, further verifying that the embryo is not a hybrid of these two species.

The genetic results coupled with the captive history of the mother make automixis the most tenable explanation for the embryo's development. This finding provides the second verified case of parthenogenesis in chondrichthyans and the first for any large-bodied species or from within the commercially important and speciose family Carcharhinidae, thus extending the known evolutionary breadth of asexual reproduction in these ancient fishes. This finding also supports the early existence of parthenogenetic abilities in vertebrates and makes it plausible that the growing number of other reported but genetically unverified cases of reproduction by a diverse range of female chondrichthyans in the extended absence of conspecific males may be the result of automictic parthenogenesis as opposed to sperm storage. Automixis in *C. limbatus* also provides a second instance of parthenogenesis in a placentally viviparous shark species, raising further questions about the relationship between placental reproduction and evolution of genomic imprinting, as has been proposed for mammals (Haig, 2004; Chapman *et al.*, 2007).

Automixis is probably rare in wild populations of *C. limbatus* with robust gender ratios. For example, assuming production of a single embryo is typical

TABLE I. Microsatellite genotypes of the mother *Carcharhinus limbatus* (M) and embryo (E). Individual allele sizes (bp) include a labelled M13 primer. Locus designations are from Keeney & Heist (2003)

Locus	M	E
*Cli*100	234/234	234/234
*Cli*13	212/232	232/232
*Cli*107	127/129	127/127
*Cli*108	150/152	152/152
*Cli*7	205/205	205/205

of automictic development in sharks (Chapman *et al.*, 2007; present study), only one out of 221 gravid female *C. limbatus* (0·45%) examined by scientists at the Natal Sharks Board in South Africa between 1978 and 2006 was documented to have been gestating a lone embryo and even this case could have resulted from processes other than parthenogenesis (*e.g.* abortion of other embryos during capture; S. Winter, pers. comm.). What is unclear, however, is whether automictic development of unfertilized ova in sharks is an occasional aberration in the ova or a facultative response of the shark to an absence of suitable mates. The latter appears to be the case in some reptiles in which some captive females have regularly switched between sexual reproduction and asexual reproduction according to the presence or absence of males (Watts *et al.*, 2006). Although this female *C. limbatus* reproduced *via* automixis during what was most likely its first ovulation and small oviparous sharks have produced several offspring in the absence of males on multiple occasions, it remains unknown whether automixis can be a repeated, facultative response to an absence of males in sharks. Regardless of how it occurs, the widespread population collapses occurring for many sharks due to overexploitation (Baum *et al.*, 2003; Baum & Myers, 2004; Robbins *et al.*, 2006; Myers *et al.*, 2007) may increase the expression of automixis if females have difficulty finding mates at low population densities and significant numbers of their ova are left unfertilized.

Whether the automictic development of unfertilized ova is selectively advantageous in sparse or strongly female-biased vertebrate populations is an open question. Although it is intuitively appealing that the ability to reproduce asexually would be selectively advantageous for females in situations where males are sparse, this may not always be the case. Automictic parthenogens have reduced genetic diversity (elevated homozygosity), with potentially reduced fitness consequences (Schuett *et al.*, 1998; Watts *et al.*, 2006; Chapman *et al.*, 2007). The genetic costs of automixis might offset the benefit of having a mechanism to avoid occasional reproductive failure in increasingly sparse, overexploited populations of large-bodied carcharhinids.

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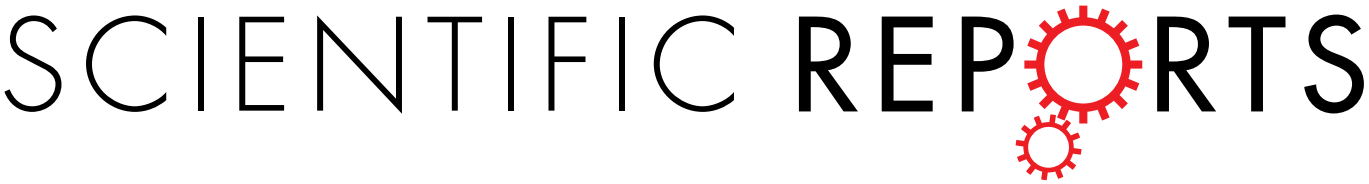

OPEN

Switch from sexual to parthenogenetic reproduction in a zebra shark

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Christine L. Dudgeon[1], Laura Coulton[2], Ren Bone[2], Jennifer R. Ovenden[1] & Severine Thomas[2,3]

Parthenogenesis is a natural form of asexual reproduction in which embryos develop in the absence of fertilisation. Most commonly found in plants and invertebrate organisms, an increasing number of vertebrate species have recently been reported employing this reproductive strategy. Here we use DNA genotyping to report the first demonstration of an intra-individual switch from sexual to parthenogenetic reproduction in a shark species, the zebra shark *Stegostoma fasciatum*. A co-housed, sexually produced daughter zebra shark also commenced parthenogenetic reproduction at the onset of maturity without any prior mating. The demonstration of parthenogenesis in these two conspecific individuals with different sexual histories provides further support that elasmobranch fishes may flexibly adapt their reproductive strategy to environmental circumstances.

Parthenogenesis is a natural form of asexual reproduction in which embryos develop in the absence of fertilisation. Occurrences of parthenogenetic reproduction in vertebrate organisms have been increasingly documented (recorded from >0.1% of extant vertebrate species)[1]. Obligate parthenogenesis, where all individuals within a species reproduce asexually, is restricted to the Squamate reptiles[2,3]. Facultative parthenogenesis, the occurrence of asexual reproduction in otherwise sexually producing species, is found more widely across major vertebrate groups including reptiles, birds, bony fish and six species of sharks and rays[1,3–12]. Mammals are an exception as facultative parthenogenesis does not naturally occur in this group due to intracellular processes such as genomic imprinting during gametogenesis[13].

Most documented cases of facultative parthenogenesis in vertebrates have been recorded from females in captive environments that have had no exposure to male conspecifics during their entire reproductive lifetime[3,6]. This raises questions regarding the adaptive strategy of facultative parthenogenesis in these isolated incidences or whether parthenogenesis in most vertebrates is accidental[14]. Novel lines of evidence can help elucidate the prevalence and function of parthenogenesis in vertebrates. In particular, parthenogenesis has been demonstrated in wild vertebrate populations: pit viper snakes[15] and sawfish[8]. Parthenogenetic offspring in these populations were identified among sexually produced offspring based on their unusually high levels of genetic homozygosity. This genetic signature in vertebrates is mostly attributed to the mechanism of terminal fusion automixis, the restoration of diploidy by fusion of the egg with a polar body[12], although gametic duplication also leads to elevated homozygosity and in most cases cannot be disregarded as the potential mechanism[3]. The presence of sexually produced litters captured from the same regions and time periods as parthenogenetic offspring suggest that complete isolation from males during a female's reproductive lifetime may not be a requirement or even a driver.

A recent study on a captive eagle ray *Aetobatus narinari* suggests that relatively short periods of separation from a potential mate may trigger a shift in reproductive strategy[9]. A single female eagle ray switched from sexual reproduction to producing a pup asexually less than one year after being separated from the male[9]. Only one other published study demonstrates this switch within an individual vertebrate. A captive *Boa constrictor imperator* produced a litter through a sexual encounter with a co-housed male *B. c. constrictor*. After a four year period of isolation she was housed with other male conspecifics during which she produced two litters. Genetic analyses demonstrated that these were comprised of parthenogenetic offspring despite what appeared to be potential mating opportunities[16]. In three other cases, captive female pythons have produced parthenogenetic offspring after having been observed copulating with male conspecifics. However, the fertility of these male snakes was not determined[3,17].

[1]The University of Queensland, Molecular Fisheries Laboratory, School of Biomedical Sciences, St. Lucia Queensland, 4072, Australia. [2]Reef HQ Aquarium, Townsville, Australia. [3]College of Marine and Environmental Sciences, James Cook University, Townsville, 4811, Queensland, Australia. Correspondence and requests for materials should be addressed to C.L.D. (email: c.dudgeon@uq.edu.au)

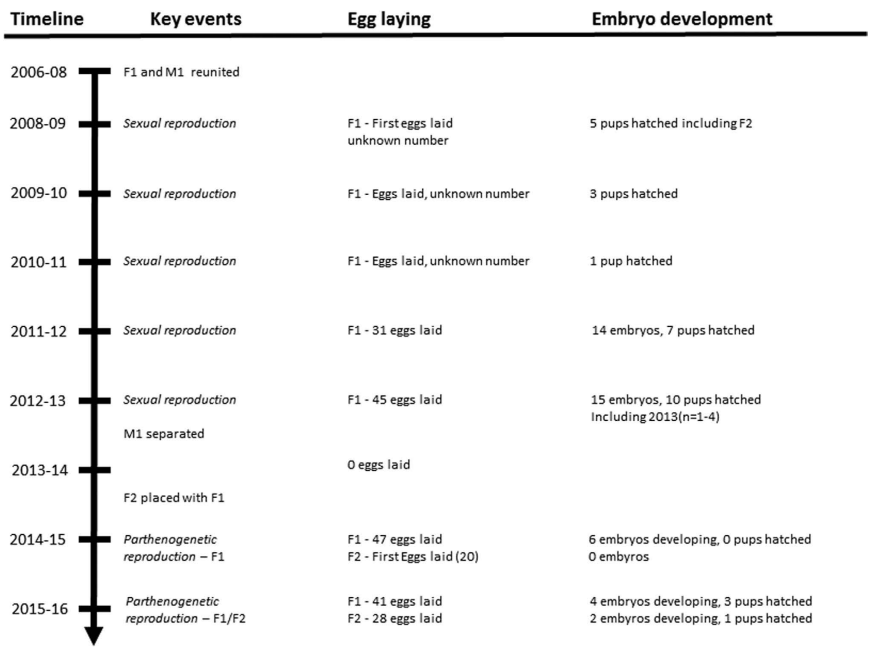

Figure 1. Timeline showing the key events of mating and separation, egg production and embryo development of sexual and parthenogenetic zebra sharks. *F1* refers to the primary mature female and *M1* to the mature male. *F2* is the sexually produced offspring of *F1* and *M2*.

Here we report on the first occurrence of an intra-individual switch from sexual to parthenogenetic reproduction in a shark species, the zebra shark *Stegostoma fasciatum*. This study is also novel in demonstrating the onset of parthenogenetic reproduction in two individual, co-housed, females with different sexual histories: parthenogenesis following sexual reproduction and without prior sexual reproduction. Zebra sharks are oviparous[7], reach maturity around 7 years of age, and live to over 35 years in captivity (pers. obs.). In 1999 a wild-captured female zebra shark (*F1*) was introduced to an already captive mature male zebra shark (*M1*) within the Reef HQ Aquarium, Townsville (Australia). The maturity of *F1* was not confirmed, however mating was attempted at this time. The pair were separated and reunited in 2006, and mating commenced at that stage. *F1* started laying eggs in 2008 and successfully produced several litters of viable offspring each year until 2013 (Fig. 1). Following mating in 2012, *M1* was separated permanently from *F1*. Offspring were produced in the breeding season spanning the austral summer (2012/2013) following this final mating event. During the next breeding season (2013/14) *F1* did not produce any eggs. At this time her immature daughter shark (*F2* born in 2009) was introduced into the same tank as her. *F1* started laying eggs again the following season (2014/15). Live embryos were observed in 6 of the 47 eggs and monitored until they were all deceased between 35 and 94 days of incubation. The daughter shark *F2* reached maturity at this time and also started laying eggs, which were visibly distinguishable from her mother's eggs due to having a slightly smaller size and thinner shell. None of *F2*'s eggs showed embryo development. During the 2015/2016 breeding season, both *F1* and *F2* laid eggs with some embryos visible for both sharks. Three juvenile sharks hatched out between February and April 2016 from the eggs of *F1*, and one juvenile shark hatched out in June 2016 from the eggs of *F2* (Fig. 1).

The presence of the embryos in the eggs of *F1* following the separation from the male could be explained by two hypotheses: (i) storage of *M1*'s sperm by *F1*, or (ii) parthenogenesis. Both hypotheses are plausible. Parthenogenesis has previously been described for this species from one captive zebra shark in the Dubai aquarium with no history of sexual reproduction[7]. *F2* was not housed with reproductively mature males at any point so only the parthenogenesis hypothesis seems plausible in her case. Although the duration of sperm storage has not been investigated in zebra sharks specifically, sperm storage for up to 45 months has been reported from a related carpet shark species[18] and the longest confirmed sperm storage of any vertebrate is recorded at 67 months in the eastern diamond-backed rattlesnake (*Crotalus adamanteus*)[19], clearly spanning beyond the period of isolation from a male that *F1* experienced. If sperm storage accounted for the offspring of *F1* in the absence of a mate, the genotypes of the offspring will reflect two-parent origin and reject the hypothesis of parthenogenesis. We employed DNA genotyping to test between these two competing hypotheses and demonstrated that *F1* switched between sexual and parthenogenetic reproductive modes quickly, skipping only one breeding season, while the daughter shark (*F2*) commenced her reproductive phase via parthenogenesis one year after maturity without any exposure to a mate. This study highlights the flexibility in reproductive strategies for elasmobranchs and we discuss the consequent ecological and evolutionary implications.

Results and Discussion

In total 14 zebra shark specific loci were scored. Nine of these loci demonstrated unique alleles that were not shared between the mother *F1* and putative father *M1* shark, and were therefore informative for parental

Ind.	Description	Parent/s	SF2		SF38		SF72		Sfa221		Sfa236		Sfa248		Sfa335		Sfa387		Sfa418	
F1	Mother		192	194	229	241	238	272	246	248	244	256	229	335	380	400	240	246	231	231
M1	Father		190	190	245	245	222	250	238	242	228	240	307	339	368	372	232	232	225	225
F2 (2009)	Sexual offspring	F1 & M1	190	194	229	245	222	238	242	248	240	256	299	307	368	380	232	240	225	231
2013:1	Sexual offspring	F1 & M1	190	194	241	245	222	272	238	246	240	256	299	339	372	400	232	246	225	231
2013:2	Sexual offspring	F1 & M1	190	192	241	245	250	272	238	246	240	256	307	335	368	400	232	240	225	231
2013:3	Sexual offspring	F1 & M1	190	192	229	245	222	238	238	246	240	256	299	307	372	380	232	246	225	231
2015:1	Parthenogenetic offspring	F1	194	194	229	229	238	238	248	248	244	256	299	299	380	380	246	246	231	231
2015:2	Parthenogenetic offspring	F1	192	192	241	241	272	272	248	248	256	256	335	335	400	400	240	240	231	231
2015:3	Parthenogenetic offspring	F1	194	194	229	229	238	238	246	246	244	256	335	335	400	400	246	246	231	231
2015:4	Parthenogenetic offspring	F1	194	194	229	229	238	238	246	246	244	256	335	335	400	400	240	240	231	231
2016:1	Parthenogenetic offspring	F1	192	192	241	241	272	272	248	248	244	244	299	299	400	400	246	246	231	231
2016:2	Parthenogenetic offspring	F1	192	192	241	241	272	272	248	248	256	256	335	335	380	380	246	246	231	231
2016:3	Parthenogenetic offspring	F1	194	194	241	241	272	272	246	246	244	244	335	335	400	400	246	246	231	231
2016:4	Parthenogenetic offspring	F1	194	194	229	229	238	238	246	246	244	244	335	335	400	400	246	246	231	231
2016:5	Parthenogenetic offspring	F2	194	194	229	229	238	238	242	242	256	256	299	299	380	380	240	240	231	231

Table 1. Genotype data at nine microsatellite loci for 15 zebra sharks *Stegostoma fasciatum* from Reef HQ Aquarium Australia. Genotypes are presented as base pair sizes. The mother shark *F1* is presented first, followed by the putative sire *M1* and the sexually produced adult offspring *F2*. The three deceased juvenile sharks from the final sexual breeding encounter are shown with the date 2013:1–3. The parthenogenetic offspring from *F1* are shown with the dates 2015:1–3 and 2016:1–4. The parthenogenetic offspring from *F2* is shown in row 2016:5. Ind. = individual.

assessment of the offspring (Table 1). For these nine loci, the offspring from the 2009 and the 2013 (n = 1–3) seasons were presumed to be of sexual origin from *F1* × *M1* and expected to demonstrate bi-parental inheritance. These individuals were heterozygous at all nine loci displaying one maternal and one paternal allele, in accordance with the sexual reproduction hypothesis. The presumed parthenogenetic offspring from *F1* (2015:n = 1–4, 2016:n = 1–3) were homozygous for one of the maternal alleles at each locus. The single offspring of *F2* (2016:5) was homozygous at all alleles that were present in *F2*'s genotype. As *F2* is the sexually produced daughter of *F1*, the alleles from eight of the nine loci also matched *F1*'s genotype. However, one locus (Sfa221) distinguished the mother of this offspring as *F2*. The offspring (2016:5) was homozygous for allele 242, which was recorded from *F2* (242, 248) and *M1* (238, 242) but not *F1* (246, 248) (Table 1).

The other five loci all demonstrated one shared allele between *F1* and *M1*. Although it is not possible to determine the parental origin of the shared allele when present in the offspring genotype, all of the parthenogenetic offspring were homozygous at each of these loci, fitting the genetic signature of parthenogenesis in elasmobranchs. The sexually produced offspring were either homozygous for the parental shared alleles or heterozygous, fitting the genetic signature of bi-parental inheritance (Supplementary Table).

These results unambiguously support the hypothesis that the embryos produced two years after the removal of the male shark were of parthenogenetic origin and not due to sperm storage. The offspring of *F2* also supported a parthenogenetic origin, demonstrating that *F2* commenced reproducing asexually in her second year of maturity. The elevated homozygosity displayed in parthenogenetic genotypes (from *F1* and *F2*) could be the genetic signature of terminal fusion automixis, which is the dominant mechanism for facultative parthenogenesis proposed for vertebrate animals[5,12,15]. In this mechanism heterozygosity is restricted to the tips of the chromosomes[12], therefore genetic signatures of randomly screened microsatellite loci tend to demonstrate elevated homozygosity. Alternative mechanisms, including gametic duplication[19] and spontaneous development of a haploid individual from an unfertilized egg[20] result in complete homozygosity[21] and cannot be ruled out[3]. However heterozygosity was observed at one locus for a parthenogenetic zebra shark in the Dubai aquarium supporting the mechanism of terminal fusion automixis in this species[7]. The analysis of *F1*'s earlier offspring born in 2009 and 2013 clearly demonstrates sexual reproduction where the offspring possess at least one allele from *M1* at each locus. This confirms that *F1* switched from sexual to parthenogenetic reproduction within a period of two years.

Van der Kooi and Schwanten[14] argued that examples of facultative parthenogenesis in vertebrates are likely to be reproductive errors and hence are indicative of accidental parthenogenesis. Under that model, asexual reproduction is rare and sporadic across species and not an adaptive strategy. Our findings suggest otherwise. Firstly we have demonstrated a relatively rapid transition from sexual reproduction to parthenogenetic reproduction in an individual animal that appears to be in response to an environmental change. Parthenogenesis was not documented from a single, isolated individual, but rather two individuals within the aquarium system with different sexual histories. Furthermore, parthenogenesis has been documented in this species from individuals captured from geographically distant locations: the western Pacific Ocean (this study) and the Red Sea[7]. Other elasmobranch and snake species have also demonstrated parthenogenetic reproduction in multiple individuals as well as across successive years in captivity[3,6,9,16,17,22]. Furthermore, the viability of a vertebrate parthenogenetic offspring has recently been demonstrated in a bamboo shark with a second generation offspring also being produced through parthenogenesis[23].

A challenge for understanding the adaptive nature of facultative parthenogenesis in elasmobranchs and other vertebrates is identifying the conditions under which it occurs. Heritability in facultative parthenogenesis has been demonstrated for poultry and *Drosophila* spp. (see review ref. 24). However sexual reproduction appears to be the dominant form of reproduction for species demonstrating facultative parthenogenesis[8,15] and therefore, it appears that internal or external cues may lead to the onset of parthenogenesis in these species. Studies in poultry found that viral infections increased the prevalence of parthenogenesis in different species, but that there were no significant effects from feed types, light levels, sex hormones or proximity to conspecifics. Increasing temperature was found to initiate the onset of parthenogenesis in silkworms and increase its prevalence in *Drosophila parthenogenata* (see review ref. 24). In this study, *F1* was kept in the same aquarium throughout minimizing any changes to her external environment. The main trigger for the switch from sexual to parthenogenetic reproduction in *F1* therefore, appears to be the removal of the mate. Similarly, the rapid transition between reproductive strategies by the eagle ray also followed the removal of the mate, supporting the hypothesis that parthenogenesis is a reproductive advantage under conditions of isolation from potential mates[12]. However this cue does not appear to be ubiquitous among vertebrates with contrasting patterns observed in snakes. A female boa constrictor demonstrated a switch from sexual to asexual reproduction, reproducing parthenogenetically in the presence of male conspecifics and not during the two intermittent years when she was housed in isolation[16,25]. Although most examples of parthenogenesis for snakes have occurred when females were isolated from mates, parthenogenesis was also documented from two captive regal pythons and one blood python following copulation with male conspecifics[3,17]. However the fertility of these male snakes has not been confirmed. To date, examples of parthenogenesis in elasmobranchs in captivity have only been reported from females isolated from males. To better understand the effect of the absence or presence of males on the onset of parthenogenesis, further studies on the genetic signatures of offspring produced from cohoused male and female individuals are also required.

It is not possible to rule out potential cues between the mother and daughter shark triggering the onset of parthenogenesis. However the female zebra shark in the Dubai aquarium was not housed with another zebra shark at any point prior to maturation and commencing parthenogenetic reproduction[7], therefore lending support more to the absence of a mate rather than the presence of another female as the driver.

Critical densities have been proposed as a driver for the onset of parthenogenetic reproduction within a species[26]. Under this scenario populations can grow to critical levels through parthenogenesis to increase downstream opportunities for mating success. However given that most examples of parthenogenesis in vertebrates from captive environments involve females kept in isolation or with few conspecifics, it is not possible to determine what a threshold would be, if at all it exists. The few examples of parthenogenesis from wild vertebrates demonstrated overall sex ratios near unity[8,15], yet this does not take into account potential spatial segregation during critical mating periods. Empirical studies in captive conditions could be undertaken to ascertain critical levels at higher densities.

The evolutionary function of facultative parthenogenesis may become clearer when mechanisms are understood across a range of taxa, but at the moment it remains debatable. Most obligate parthenogenetic vertebrates arise from hybridization between closely related species, resulting in elevated individual heterozygosity relative to the parental genotypes[11,27,28]. This is considered adaptive for colonizing new areas where high genetic diversity may provide the necessary genetic tools to adapt to new conditions[29]. Although most obligate parthenogenetic lineages are short lived and therefore considered of greater ecological than evolutionary importance[11], they may have long-term evolutionary adaptive advantages where back-crossing with sexual species enables genera to expand phylogenetically and geographically[27]. In contrast, facultative parthenogenesis results in greatly reduced genetic diversity and presumably less adaptive advantage in dealing with novel environmental conditions. The accumulation of deleterious mutations (Muller's ratchet[30]) results in lineages being short lived unless there is the capacity for sexual reproduction. Sexual reproductive competency of parthenogenetic offspring has not yet been demonstrated in vertebrates though it has been recorded from other organisms (e.g. *Drosophila*[31]).

An interesting point of difference in facultative parthenogenesis between elasmobranchs and other vertebrate species is the consequence of the genetic mechanism for sexual determination. Cytogenetic analysis of a subset of elasmobranch species demonstrated XY male heterogamety and XX female homogamety similar to mammals[32]. This contrasts with birds and many reptiles, which demonstrate ZW female heterogamety with ZZ male homogamety. The exception is the basal snake lineages which may produce viable WW female offspring[25]; however see Booth & Schuett[3] where it is suggested that basal snakes including the Pythons and Boas may actually possess XX/XY sex chromosomes as opposed to the commonly accepted ZZ/ZW system. Facultative parthenogenesis may be particularly advantageous for species having ZZ male homogamety, as it leads to the production of males, which are potential future mates. In elasmobranchs however, all observed viable offspring produced by facultative parthenogenesis are female[6,7,9].

Facultative parthenogenesis leading to female offspring may then have the adaptive advantage of a 'holding on' mechanism, through maintaining female lineages until potential male mates become available again following immigration. In particular, elasmobranchs are considered to have ancient lineages with many species extending millions of years back in the fossil records[33]. Population genetic analysis of several elasmobranch species has revealed signatures of population bottlenecks associated with glaciation periods[34,35]. Facultative parthenogenesis may have assisted populations to survive through these periods of isolation. To address these ideas it's important to identify more examples of facultative parthenogenesis from the wild. Although the exact mechanisms triggering facultative parthenogenesis currently remain a mystery, the reproductive flexibility it potentially provides for vertebrates may be underestimated for species' survival and evolution. Examination of contemporary isolated populations as well as empirical studies with captive individuals will help investigate the mechanisms, functions and prevalence of facultative parthenogenesis in vertebrate species.

Methods

Tissue samples for DNA analysis were collected during husbandry procedures from the mother shark (*F1*); the putative father shark (*M1*); the mature daughter shark *F2* (hatched 2009 from reproduction between the two former individuals); four of the deceased embryos from *F1* in the austral summer 2014/15 season (2015:1–4); 3 hatchlings and 1 deceased embryo from *F1* in the 2015/16 season (2016:1–4); and 1 hatchling from *F2* (2016:5). To assess the timing of the switch between sexual and parthenogenetic reproduction in *F1*, we also sampled three offspring that had hatched but died during juvenile stages from the last breeding season where the female and male were cohoused (2013:1–3). All methods were carried out in accordance with relevant guidelines and regulations following husbandry procedures within the Reef HQ Aquarium, Townsville and with approval by the University of Queensland Animal Ethics Committee (#ZOO/ENT/490/05).

DNA was extracted and genotyped at 14 microsatellite loci developed specifically for zebra sharks (as per refs 36 and 37). Genotypes were scored using Geneious version 9.1.3[38].

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